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 only 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 genomic probes (D'Hont et al., 2000; Mukai and Nakahara, 1993; Shishido 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 IBA and 0.2% (w/v) Gelrite. Cultures were maintained at 27 °C in darkness for the first 10 d and transferred to a 16-h light (60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and 8-h dark regimen. Young roots 1 to 2 cm long were harvested and immersed in 2 mM 8-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 Na_2EDTA (pH 4.0).

Probe preparation. Total DNA of *D. kaki* and *D. glandulosa* were 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 by the High Prime DNA labeling system according to the supplier's instructions (Boehringer Mannheim, Tokyo).

Multi-color genomic in situ hybridization (MCGISH). Prior to MCGISH, chromosome samples on the slides were treated with 100 $\text{mg}\cdot\text{mL}^{-1}$ RNase A in 2 $\times$ SSC (0.3 M NaCl and 0.03 M sodium citrate) at 37 °C for 60 min. They were washed with 2 $\times$ 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% formamide in 2 $\times$ SSC. 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 denatured for 10 min on a heat block at 80 °C and then hybridized for 10 d at 37 °C. After hybridization, they were washed twice in 2 $\times$ SSC, once in 50% formamide in 2 $\times$ SSC, and once in 4 $\times$ SSC, each for 10 min at 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 (1%, Boehringer Mannheim) in 1% BSA in BT buffer was dropped onto the chromosome samples on the slide. After the slide was incubated at 37 °C for 60 min, the avidin-rhodamine solution was rinsed with BT buffer at 42 °C three times 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 and briefly washed three times with BT buffer at 42 °C for 5 min each. The samples were blocked as described above and avidin-rhodamine (1%, w/v) and anti-DIG-FITC (10%, w/v; Boehringer Mannheim) in 1% BSA in BT buffer was dropped onto the chromosome samples on the slide to detect biotin- and DIG-labeled probes simultaneously. Then the chromosome samples on the slide were incubated at 37 °C for 60 min and rinsed twice with BT buffer and once with 2 $\times$ SSC at 42 °C for 10 min each. The chromosome samples were counterstained with 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ 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]

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Fig. 1. Multi-color in situ hybridization (MCGISH) of the metaphase of a somatic hybrid between *D. kaki* and *D. glandulosa*. The bar represents 10 μ m. (A) Photograph of MCGISH using total DNA probes from *D. kaki* and *D. glandulosa*. The total DNA probe from *D. kaki* was detected with rhodamine and that from *D. glandulosa* was detected with FITC. Chromosomes from *D. kaki* revealed reddish orange color while those from *D. glandulosa* did greenish yellow. Arrows indicate cross-hybridization with both probes. (B) Schematic representation of Fig. 1A showing the origin of each chromosomes. Those from *D. kaki* are drawn in white, while those from *D. glandulosa* are black.

octane (DABCO) and 1% (w/v) p-phenylenediamine (PPD) in phosphate buffer (pH 7.4). The chromosome samples were observed with a fluorescence microscope (Axiophot, Zeiss, Tokyo) with a high-sensitivity cooled CCD camera (PXL 1400, Photometrics, Tokyo). The B- and G-light excitation filters were used for the detection of FITC and rhodamine, respectively. The signal images were analyzed by imaging software (IPLab Spectrum 3.1, Signal Analytics, Tokyo).

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 ≈ 5 years. Even after 5 years of subcultures, all 120 chromosomes were retained (Fig. 1).

MCGISH distinguished between the parental chromosomes of *D. kaki* and *D. glandulosa* (Fig. 1 A, B). Ninety chromosomes from *D. kaki* that hybridized with biotin-labeled probes showed reddish-orange fluorescence when detected with avidin-rhodamine. Thirty chromosomes from *D. glandulosa* that hybridized with DIG-labeled probes showed greenish-yellow fluorescence when detected with anti-DIG-FITC. Some chromosomes cross-hybridized with both probes at their terminal or other parts (Fig. 1A, arrows).

Somatic hybridization is a major tool for

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.

Literature Cited

- Babiychuk, E., S. Kushnir, and Y.Y. Gleba. 1992. Spontaneous extensive chromosome elimination in somatic hybrids between somatically congruent species. *Theor. Appl. Genet.* 84:87–91.
- D'Hont, A., A. Paget-Goy, J. Escout, and F. Carreel. 2000. The interspecific genome structure of cultivated banana, *Musa* spp., revealed by genomic DNA in situ hybridization. *Theor. Appl. Genet.* 100:177–183.
- Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bul.* 19:11–15.
- Escalante, A., S. Imanishi, M. Hossain, N. Ohmido, and K. Fukui. 1998. RFLP analysis and genomic in situ hybridization (GISH) in somatic hybrids and their progeny between *Lycopersicon esculentum* and *Solanum lycopersicoides*. *Theor. Appl. Genet.* 96:719–726.
- Fahleson, J., U. Lagercrantz, A. Mouras, and K.

- Glimelius. 1997. Characterization of somatic hybrids between *Brassica napus* and *Eruca sativa* using species specific repetitive sequences and genomic in situ hybridization. *Plant Sci.* 123:133–142.
- Fukui, K. 1996. Plant chromosomes at mitosis, p. 1–18. In: K. Fukui and S. Nakayama (eds.). *Plant chromosomes: Laboratory methods*. CRC Press, New York.
- Hizume, M. 1994. Allodiploid nature of *Allium wakegi* Araki revealed by genomic in situ hybridization and localization 5S and 18S rDNAs. *Jpn. J. Genet.* 69:407–415.
- Jacobsen, E., J.H. De Jong, S.A. Kamstra, P.M.M.M. Van Den Berg, and M.S. Ramanna. 1995. Genomic in situ hybridization and RFLP analysis for the identification of alien chromosomes in the backcross progeny of potato (+) tomato fusion hybrids. *Heredity* 74:250–257.
- Kitamura, S., M. Inoue, N. Ohmido, and K. Fukui. 1997. Identification of parental chromosomes in the interspecific hybrids of *Nicotiana rustica* L. x *N. tabacum* L. and *N. glauca* Domin x *N. tabacum* L., using genomic in situ hybridization. *Breeding Sci.* 47:67–70.
- Mukai, Y. and Y. Nakahara. 1993. Simultaneous discrimination of the three genomes in hexaploid wheat by multi-color fluorescence in situ hybridization using total genomic and highly repeated DNA probes. *Genome* 36:489–494.
- Nakamura, Y. and S. Kobayashi. 1994. DNA restriction fragment length variability in *Diospyros kaki* and related *Diospyros* species. *HortScience* 29:809–811.
- Ng, F.S.P. 1978. *Diospyros roxburghii* and the origin of *Diospyros kaki*. *Malaysian Forester* 41:43–50.
- Raina, S.N., Y. Mukai, and M. Yamamoto. 1998. In situ hybridization identifies the diploid progenitor species of *Coffea arabica* (Rubiaceae). *Theor. Appl. Genet.* 97:1204–1209.
- Shishido, R., S. Apisitwanichi, N. Ohmido, Y. Okinaka, K. Mori, and K. Fukui. 1998. Detection of specific chromosome reduction in rice somatic hybrids with the A, B, and C genomes by multi-color genomic in situ hybridization. *Theor. Appl. Genet.* 97:1013–1018.
- Tamura, M., R. Tao, and A. Sugiura. 1998. Production of somatic hybrids between *Diospyros glandulosa* and *D. kaki* by protoplast fusion. *Plant Cell Tissue Organ Cult.* 54:85–91.
- Yonemori, K., S. Kanzaki, D.E. Parfitt, N. Utsunomiya, S. Subhadrabandhu, and A. Sugiura. 1998. Phylogenetic relationship of *Diospyros kaki* (persimmon) to *Diospyros* spp. (Ebenaceae) of Thailand and four temperature zone *Diospyros* spp. from an analysis of RFLP variation in amplified cpDNA. *Genome* 41:173–182.
- Zhuang, D.H., A. Kitajima, M. Ishida, and Y. Sobajima. 1990. Chromosome numbers of *Diospyros kaki* cultivars. *J. Jpn. Soc. Hort. Sci.* 59:289–297.