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BRIEF COMMUNICATION

Chromosomal location of 45S rDNA and *dfr* gene in *Citrus sinensis*

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Abstract

Dihydroflavonol 4-reductase (DFR) is a key enzyme in the anthocyanin biosynthesis. In this study, the localization of 45S rDNA and *dfr* gene (named as *CsDFR-bo*) of the blood orange (*Citrus sinensis* Osbeck cv. Ruby) on chromosomes was investigated by fluorescence *in situ* hybridization (FISH). A karyotype of *C. sinensis* was reconstructed based on the length of mitotic metaphase chromosomes. The 45S rDNAs were localized on chromosomes 2p and 7q. The detection ratios of 45S rDNA in the two chromosomes were 69.5 and 77.3 %, respectively. The *CsDFR-bo* was proved to be a single-copy gene and localized on chromosome 3p. The detection ratio of *CsDFR-bo* was 8.2 %.

Additional key words: anthocyanin biosynthesis, dihydroflavonol 4-reductase gene, fluorescence *in situ* hybridization.

Anthocyanins are red and purple flavonoid pigments. Their synthesis has been studied extensively by both biochemical and genetic methods, and most of the enzymes involved in the synthetic pathway and their respective genes have been characterized (Cone 2007, Ahmed *et al.* 2009). Dihydroflavonol 4-reductase (DFR) is the first specific enzyme in anthocyanin biosynthesis. The full length of *dfr* gene was obtained from *Citrus sinensis* Osbeck. Southern blot analysis of genomic DNA indicated that *dfr* gene was present as a single copy in the cultivars Tarocco, Navel and Ovale (Lo Piero e*t al.* 2006).

45S, 5.8S, 18S and 25S rDNAs were extensively studied in plant genomes. 45S rDNA was highly conserved and located in one or more clusters of chromosomes (Pedersen e*t al.* 1994). Because of their easy visualization by fluorescence *in situ* hybridization (FISH), these rDNA genes are excellent cytological markers for karyotype analysis, especially in species with many small and equal chromosomes. However, the physical mapping of low- or single-copy sequences on metaphase chromosomes is still difficult, because the debris of cell wall and cytoplasm reduces the accessibility of target DNA and increases background, and consequently results in a relatively low signal-to-noise ratio (Lehfer *et al.* 1993, Jiang *et al.* 1995). Nevertheless, successful detection of single-copy genes has been reported by several laboratories. For example, Fransz *et al.* (1996) mapped the chalcone synthase A gene on *Petunia hybrida* mitotic metaphase chromosomes with a 1.4-kb probe.

In this study, blood orange (*Citrus sinensis* Osbeck cv. Ruby) was obtained from Citrus Research Institute, Chinese Academy of Agricultural Sciences in Chongqing. Seeds were peeled off and cultured on Murashige and Skoog (1962; MS) medium. The 3-week-old seedlings were used in this experiment.

According to the cetyltrimethylammonium bromide (CTAB) method (Sambrook *et al.* 1989), genomic DNA was isolated from fresh leaves. One pair of primers FDFR (5'-GGGCTCTATAGCTGAGA-3') and RDFR (5'-GGA TACTTCGCTGACAT-3') were synthesized and used to amplify the core fragment of *dfr* gene by PCR at an annealing temperature of 45 °C. The amplified fragment was cloned into *pGEM T-easy* vector (*Promega*, Madison, WI, USA) and transformed into *Escherichia coli* strain DH5α, followed by sequencing. The DNA probes for *CsDFR-bo* (a 1.6-kb core fragment) and 45S rDNA were labeled with the *DIG* DNA labeling kit (*Roche*, Basel, Switzerland), following the supplier's instructions.

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Abbreviations: DFR - dihydroflavonol 4-reductase; FISH - fluorescence *in situ* hybridization.
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Somatic cells were obtained from leaf buds of the seedlings and pretreated for 3 h in saturated *P*-dichlorobenzene solution at 25 °C, fixed in ethanol: acetic acid, 3:1 (v/v), and stored at 4 °C. Chromosome preparations were made by macerating the leaf buds in enzyme solution (2 % cellulase *Onozuka*, 2 % pectolyase *Y-23*, 1 mM EDTA, pH 4.2) at 37 °C for 1 h. After washing with distilled water, the digested meristematic parts were transferred to glass slides, spread on a drop of fresh fixative (glacial acetic acid : ethanol, 1:3) with a pair of tweezers, and then air-dried. The chromosomal spreads were examined by phase contrast microscopy (*Axiophot*, *Zeiss*, Jena, Germany) before the FISH analyses were conducted.

The FISH procedure was done as described by Li and Arumuganathan (2001) with some modifications. The slides were treated at 60 °C for 1 h, denatured in 70 % deionized formamide solution at 70 °C for 2.5 min, dehydrated in an ethanol series (70, 95 and 100 % at -20 °C), and air-dried. The hybridization mixture was composed of 50 % formamide, 20 % dextran sulphate, 10 % of 2× SSC, 10 % 50 μg cm^{-3} of salmon sperm DNA, 10 % 5 μg cm^{-3} of probe DNA and 0.02 % SDS. It was denatured at 75 °C for 5 min and quenched on ice at least 5 min. Approximately 0.03 cm^3 of denatured probe mixture was applied per slide. The slides were covered with cover slips (24 × 32 mm) in a humid chamber at 37 °C for 24 - 48 h.

After hybridization, the cover slips were removed carefully and the samples were washed in 2× SSC (10 min) at room temperature, 2× SSC (10 min) at 37 °C, 1× PBS (5 min) at room temperature. For detection of the probe, a slide was applied with 5 μg cm^{-3} of anti-digoxin-FITC (*Roche*) in detection buffer containing 0.5 % BSA, incubated at 37 °C for 45 min and washed three times (5 min each) in 1× PBS. For amplification of the fluorescence signal, the slides were treated with 5 μg cm^{-3} of rabbit anti-sheep-FITC (*Vector Laboratories*, Peterborough, UK) in 0.5 % BSA solution at 37 °C for 45 min. After incubation, the slides were washed three times in 1× PBS, and air-dried.

The slides were mounted in an anti-fade solution containing 1 μg cm^{-3} of 4',6-diamidino-2-phenylindole (DAPI) to reduce fading of the fluorescence. Chromosomes were examined with an *Olympus BX60* (Tokyo, Japan) fluorescence microscope equipped with *Sensys 1400E* cooled CCD camera. Green and blue images were captured in dark, respectively. The images were then combined and pseudo-coloured using the software *Metamorph 4.6.3* (*Universal Imaging Corp.*, West Chester, PA, USA).

The 141 metaphase cells were used to count the numbers of the 45S rDNA signals. The results showed that the 45S rDNA probes produced three specific signals on metaphase chromosomes (Fig. 1. *A,B*). A banding-pattern karyotype was obtained by using a new method of $CMA^+/DAPI^-$ banding pattern by Pedrosa *et al.* (2000). The karyotype of *C. sinensis* was reconstructed based on the length of mitotic metaphase chromosomes. Five metaphase cells which were dispersed well had been investigated for karyotype analysis. The results showed that one pair of 45S rDNA loci were located on the short arm, close to the centromere constriction, of chromosome 2, and another pair of 45S rDNA loci were localized to the proximate telomere on the long arm of chromosome 7 (Fig. 1*A,B*). The arm ratio of chromosome 2 was 1.39 ± 0.04, and the arm ratio of chromosome 7 was 1.42 ± 0.22. The detection ratios of 45S rDNA in 2p chromosomes were 69.5 % while the detection ratios of 45S in 7q chromosomes were 77.3 %.

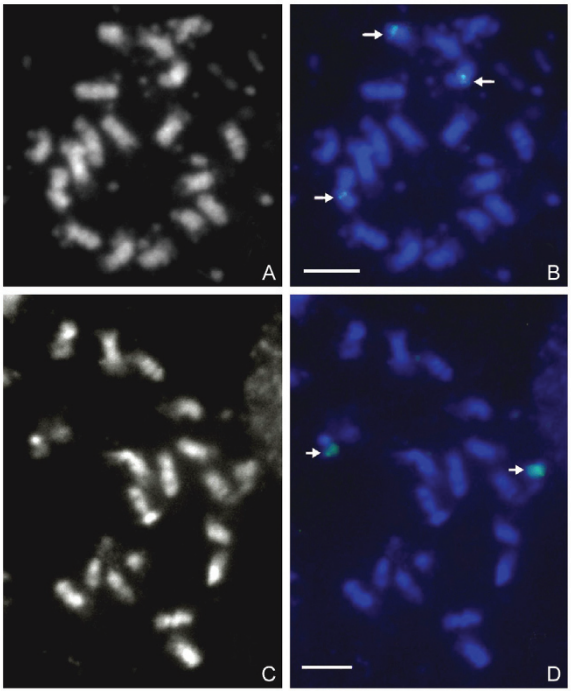

Fig. 1. Fluorescence *in situ* hybridization (FISH) mapping of 45S rDNA and *dfr* gene on the mitotic metaphase chromosomes of *C. sinensis* cv. Ruby: *A* - metaphase chromosomes, *B* - a merged image of the metaphase chromosomes and the FISH signals of 45S rDNA, C - metaphase chromosomes, *D* - a merged image of the metaphase chromosomes and the FISH signals of *dfr* gene. The *white arrows* point to the loci of the genes. *Bar* = 5 μm.

The chromosome distribution of 45S rDNA loci was characterized in six citrus cultivars: Comuna, Hamlin, Olivelands, Parson Brown, Pera and Valencia. No difference in position or size of the 45S rDNA clusters was observed among these six cultivars (Pedrosa *et al.* 2000). Out of three sites of 45S rDNA, two are proximally localized to 2p chromosomes, while the other one is terminally localized to one chromosome of 7q in the suggested model for these six cultivars. Our result confirmed the number and location of 45S rDNA sites in *C. sinensis* cv. Ruby.

High-resolution cytogenetic maps provide important biological information on genomic organization and

function, because they correlate genetic distance with cytological structures, and are an invaluable complement to physical sequence data (Wang *et al.* 2006). However, the detection of low-copy DNA sequences that are shorter than 2 kb on plant chromosomes is still difficult by FISH.

We found that it was better to use the chromosome spreads that were freshly prepared. Extended time of hybridization could increase the strength of signals, too. For the detection of *dfr* gene which is a single-copy gene in *C. sinensis* by FISH, 48 h of hybridization time was used, which was two times as long as for 45S rDNA. The signals of *dfr* gene were clearly detected (Fig. 1*D*). The 341 metaphase cells were used to count the numbers of the *CsDFR-bo* signals. The *CsDFR-bo* probes hybridized two specific locations on metaphase chromosomes (Fig. 1*C,D*). The detection ratio of *CsDFR-bo* was 8.2 %. The loci of *dfr* gene were localized to the proximate telomere on short arms of both homologues of chromosome 3 (Fig. 1*C,D*). The arm ratio of chromosome 3 was 1.30 ± 0.13. The result showed that the *dfr* gene was a single-copy gene in *C. sinensis*. This result is consistent with the Southern-blot analysis, which found that there was only one copy of *dfr* gene in the genome of *C. sinensis* (Lo Piero *et al.* 2006).

In conclusion, 45S rDNA and *dfr* gene were accurately localized on the chromosomes of *C. sinensis* Osbeck cv. Ruby by FISH. The result showed that there was only one copy of *dfr* gene in the genome of *C. sinensis* Osbeck cv. Ruby. We also reconstructed the karyotype model of *C. sinensis* Osbeck by the length of the chromosomes, to which the gene loci of 45S rDNA and *dfr* gene were marked.

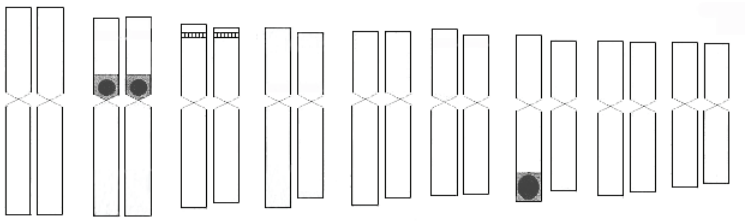

Fig. 2. Schematic representation of the 18 chromosomes in *C. sinensis* cv. Ruby based on the lengths of chromosomes. Grey areas with black dots were 45S rDNA loci and bands with strips were *CsDFR-bo* gene loci.

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