A Chromosome Preparation Method Using Young Leaves of Citrus

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Summary

A useful method of preparing chromosomes for identification was developed by using young citrus leaves.

The preparatory procedures are as follows: 3–5 mm long young leaves were enzymatically macerated; the cell suspension in a hypotonic solution was dropped onto a glass slide from about 30 cm height. The preparations were stained with Giemsa. For the enzymatic maceration, incubating young, finely minced leaves of ‘Tosa-Buntan’ pummelo (*C. grandis* [L.] Osb.) in a mixture of 0.3% cellulase and 0.2% pectolyase for 120 to 180 min at 37°C was optimum for chromosome preparation because it allowed morphological characteristics to become visible.

The present method using young leaves was considered as a useful chromosome preparation method for chromosome identification in citrus.

Key Words: chromosome, citrus, young leaf.

Introduction

Recently studies on linkage maps in citrus (Cai et al., 1994; Fang et al., 1998a) and classifications (Yamamoto and Kobayashi, 1996; Coletta Filho et al., 1998; Fang et al., 1998b) have been conducted based on DNA analysis which has accelerated chromosome research involving taxonomy and the genome.

Chromosome research of fruit trees has used the squash method for analysis. Oiyama (1981) developed the squash method for citrus chromosome preparation which has been suitable for the investigation of chromosome numbers, but not for chromosome identification by their morphology. Morphological classification of rice chromosomes, whose sizes are small, similar to those of citrus, was developed by using an enzymatic maceration method for chromosome preparation (Kurata and Omura, 1978) but this chromosome classification system based on morphological characters was established by Iijima et al. (1991). The enzymatic maceration method has been applied to fruit trees (Omura, 1988; Zhuang et al., 1990; Ito et al., 1992). Zhuang et al. (1990) reported that an enzymatic maceration method was more suitable for chromosome preparation than the squash method. But, current method of enzymatic maceration requires highly skilled personnel to prepare a good spread of chromosomes.

Recently, research on human chromosomes has

progressed rapidly, leading to the following procedure: the cultured cells are treated hypotonically for cell expansion; the cells are suspended in a fixative solution and then dropped onto a glass slide (Yoshida, 1994).

In plants, root tips of the seedlings have been used for preparing chromosome samples but the aerial tissues, e.g., young leaves are more desirable for accurate cytological and karyotyping studies in heterozygous fruit trees.

We developed a preparation method, using young citrus leaves, which includes the enzymatic maceration, cell suspension, hypotonic treatment and the dropping of suspended cells onto a glass slide.

Materials and Methods

Basic procedures of chromosome preparation

Young leaves (3–5 mm length) are immersed in 2mM 8-hydroxyquinoline for 3 hrs at 20°C, fixed in a fixative solution (methanol: acetic acid =3:1, v/v) and stored at 5 °C until used. Fixed leaves are washed in distilled water, finely cut, and immersed in Cellulase Onozuka RS (Yakult Co. LTD.) and Pectolyase Y-23 (Kikkoman Corp.) within a hypotonic solution (75 mM KCl+7.5 mM Na₂-EDTA, pH 4.0). A finely cut leaf in a 1.5ml tube with 100 µl of enzyme solution is incubated at 37 °C. After incubation, the solution containing macerated cells is suspended by pipetting with gently mixing, and 900 µl of the hypotonic solution is added, and kept for one hour. The cell suspension is centrifuged at 1500 rpm for 5 min and 900 µl of the supernatant is gently removed by using a micro-pipette; and the residue is washed and centrifuged twice with the fixative solution as before. After the last rinse, 60 µl of residue is

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suspended and dropped from 30 cm height onto a glass slide. After the slides are air-dried overnight and stained with 2% Giemsa solution for 15 min, the chromosomes are observed with a microscope (OLYMPUS BX-50), photographed with an OLYMPUS PM-20, and then evaluated.

Conditions of enzymatic maceration

In April, young leaves were collected from 'Tosa-Buntan' pummelo (*C. grandis* [L.] Osb.) trees, planted at the Faculty of Agriculture, Kochi University. A preliminary experiment on enzymatic maceration used 1, 1/2, 1/5, 1/10, 1/20, 1/50, and 1/100 strengths of 3% cellulase and 1% pectolyase and incubated for 120 min at 37°C. After an evaluation of their cell status, 8 slides 1, 1/5, 1/10, and 1/20 strengths were used for the chromosome evaluation. Secondly, cellulase (0.15, 0.3, 0.6%) × pectolyase (0.1, 0.2%) and the duration of 120, 150, and 180 min at 37°C were tested, using 6 slides. Finally, cellulase (0.2, 0.3%) × pectolyase (0.1, 0.2, 0.3%) and the duration of 180 and 240 min at 37°C were tested, using 4 slides. The chromosomes were visually evaluated into 4 grades: 3=relatively well-defined chromosomes, 2=isolated chromosomes difficult to evaluate, 1=cells well macerated but few chromosomes isolated, 0=poorly macerated cells; no chromosomes.

Results and Discussion

The cells macerated with 1, 1/10, and 1/100 strength of 3% cellulase + 1% pectolyase in Fig. 1 reveals that at 1 strength, there were small numbers of isolated cells and many clumps of cells with smooth surfaces that seemed to be digested well. At 1/5, 1/10, and 1/20 strengths, many cells were sufficiently isolated and many chromosomes were observed without their cytoplasm. At 1/100 strength, cells were isolated to some degree, but the chromosomes were observed with the stained cytoplasm in the background. Better chromosome evaluations were obtained at 1/5, 1/10, and 1/20 strengths than at 1 strength (Table 1).

Out of the combinations of cellulase (0.15, 0.3, 0.6%)

Table 1. Effects of enzyme concentrations for 120 min treatment on young 'Tosa-Buntan' pummelo leaves for chromosome preparation.

Enzyme concentration		Score ^z
Cellulase+Pectolyase	Strength	
3%+1%	1	1.0
0.6%+0.2%	1/5	2.5
0.3%+0.1%	1/10	2.4
0.15%+0.05%	1/20	2.1

^z 0: Poorly macerated cells; no chromosomes.

1: Cells well macerated but few chromosomes isolated.

2: Isolated chromosomes difficult to evaluate.

3: Relatively well-defined chromosomes.

Fig. 1. Photomicrograph of macerated cells of young 'Tosa-Buntan' pummelo leaves by 1(A), 1/10(B) and 1/100(C) strengths of 3% cellulase + 1% pectolyase. Bar indicates 100 μm.

× pectolyase (0.1, 0.2%) for 120, 180, and 240 min, high scores were attained with 0.3% cellulase + 0.2% pectolyase for 120 and 180 min, as well as in 0.15% cellulase + 0.2% pectolyase for 180 min (Table 2).

Among the combinations of cellulase (0.2, 0.3%) × pectolyase (0.1, 0.2, 0.3%) for 180 and 240 min, those of 0.2 or 0.3% cellulase + 0.2% pectolyase for 180 and 240 min had high scores (Table 3). Incubation in 0.3% cellulase + 0.2% pectolyase for 180 min yielded reproducible chromosome preparations in which morphological characteristics were discernible (Fig. 2.).

Incubation of fine cut citrus leaves in 0.3% cellulase +

Table 2. Effects of low enzyme concentrations and treatment times on young 'Tosa-Buntan' pummelo leaves for chromosome preparation.

Enzyme concentration	Treatment time (min)		
	120	180	240
Cellulase+Pectolyase			
0.6%+0.2%	0.0 ^z	1.8	2.0
0.6%+0.1%	0.5	1.7	1.7
0.3%+0.2%	2.5	2.5	2.3
0.3%+0.1%	1.0	2.2	2.2
0.15%+0.2%	1.7	2.5	2.0
0.15%+0.1%	1.2	1.7	2.3

^z Score; see Table 1.**Table 3.** Enzyme combinations and concentrations used for isolating chromosome from young 'Tosa-Buntan' pummelo leaves.

Enzyme concentration	Treatment time (min)	
	180	240
Cellulase+Pectolyase		
0.3%+0.3%	2.5 ^z	3.0
0.3%+0.2%	3.0	3.0
0.3%+0.1%	2.3	2.3
0.2%+0.3%	2.8	3.0
0.2%+0.2%	3.0	3.0
0.2%+0.1%	1.5	1.5

^z Score; see Table 1. **Fig. 2.** Photomicrograph of chromosomes from young 'Tosa-Buntan' pummelo leaves incubated in 0.3% cellulase + 0.2% pectolyase for 180 min. Bar indicates 5 μ m.

0.2% pectolyase for 120 or 180 min at 37°C resulted in suitable preparations precise enough for chromosome identification. Compared with the enzymatic maceration method of citrus root tips by Guerra (1993) and Miranda et al. (1997) utilizing 2% cellulase + 2% pectinase for 30 min and 2% cellulase + 6% pectinase for 20 min,

respectively, and that of 2 to 4% cellulase + 6% pectolyase for 120–150 min and 2% cellulase + 1.5% pectolyase + 0.3% macerozyme for 60–90 min by Ito et al. (1992) and Matsuyama et al. (1996) respectively, our use of lower enzyme concentrations with a longer incubation period yielded a better chromosome preparation.

Our pre-fixation treatment with 2 mM 8-hydroxyquinoline solution for 3 hr at 20°C allowed prometaphase or metaphase chromosomes to become visible. In our additional observation of the pre-fixation treatments with 2, 10, 20 mM 8-hydroxyquinoline solution for 0, 3, 6, 9 hr at 20°C, the treatments with 2, 10, 20 mM for 0, 3, 6 hr were also effective for chromosome preparation by using young leaves.

When leaves were less than 3 mm in blade length, chromosomes were well-spread but frequently not visible, whereas with leaves longer than 6 mm, the chromosomes were not distributed well and some cytoplasm remained. Hence, leaf size and the fineness of the initial mincing also influence the degree of enzymatic maceration.

In the enzymatic maceration method (Kurata and Omura, 1978; Zhuang et al., 1990; Fukui and Iijima, 1991), a skillful tapping of the slide with forceps is necessary to prepare a good spread of chromosomes, whereas, the same results are achieved in our method by dropping suspended cells from a 30 cm height.

Root tips of seedlings were used for preparation in most citrus chromosome research (Oiyama, 1981; Ito et al., 1992; Guerra, 1993; Matsuyama et al., 1996; Miranda et al., 1997). Since many kinds of citrus are polyembryonic and produce nucellar seedlings, it is necessary to distinguish whether the seedlings are zygotic or not. In pummelo, a monoembryonic citrus, we found that the chromosome compositions based on the CMA band pattern in the seedlings were different from that of the seed or pollen parent (unpublished). This indicates that the chromosome compositions of zygotic seedlings in other fruit trees may not be the same as that of the mother tree. In many fruit trees, root tips are difficult to collect from the mother tree, whereas young leaves are easily accessible for chromosome preparation. Hence, our method of steeping small young leaves in a pre-fixation solution, followed by a modified enzymatic maceration should be applicable for chromosome research in many kinds of fruit trees.

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カンキツの幼葉を用いた染色体標本作製技術

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摘 要

カンキツの幼葉を用い、染色体の識別に有効な染色体標本作製法を検討した。染色体標本作製は、3~5 mmの幼葉を用い、組織を酵素解離した後に細胞を懸濁して低張処理を行い、懸濁細胞を30 cm程度の高さからスライドガラスに滴下する方法で行い、ギムザ染色した。‘土佐文旦’幼葉を用いて酵素

処理条件を検討した結果、0.3% セルラーゼ + 0.2% ペクトリアーゼの37℃、120分または180分間処理において、広がり良好な染色体像が安定して得られ、染色体の形態的な特徴が観察された。