Cell Research(1995),5,1–7

Chromosomal localization of ribosomal DNA sequences in an apple rootstock using a digoxygenin detection system[1]

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ABSTRACT

A 6kb rDNA probe comprising the 18S coding plus spacer sequences has been hybridized to the metaphase chromosomes of apple rootstock cultivar MM106 demonstrating the localization of ribosomal gene arrays in the vicinity of the telomeric regions of the short arms of chromosomes 6 and 14. The in situ results using digoxygenin labelling coupled to an alkaline phosphatase immunoassay were confirmed by silver staining for NORs and nucleoli. This study demonstrates the feasibility of molecular cytogenetic analysis of very small chromosomes(1.0-2.7 μm) of apple.

Key words: *Malus, chromosomes, in situ hybridization, karyotype analysis, silver staining.*

INTRODUCTION

Our group is currently developing non-radioactive systems for the *in situ hybridization* of DNA markers to the chromosomes of apple (*Malus* sp.) in order to assign marker linkage groups to physical chromosomes and aid mapping of the apple genome[1-3]. Determination of the location of genes and repeat sequences by *in situ*

1. Dedicated to Professor Zhen YAO.

hybridization of biotinylated probes to metaphase preparations has been reported in several plant species with larger chromosomes than *Malus* including *Allium*[4], *Pisum*[5, 6], *Crdpis*[7], *Picea*[8] and *Triticum*[9-11]. However, there has been limited work so far on plant species with very small chromosomes where cytogenetic analysis is more diffcult. There are two recent reports on karyotype analysis for Arabidopsis which has chromosomes of comparable size but fewer in number (2n=10) than Malus (2n=34)[12, 13]. This group used digoxygenin labelling coupled with a fluorescent detection system to determine the chromosomal localization of rDNA (9kb probel and of a smaller tandem repeat sequence(380bp probe). Although there is a recent report[8l of successful in situ hybridization to chromosomes of a woody gymnosperm (Picea), there are no such reports for woody angiosperms.

This paper describes the use of digoxygenin-labelled apple rDNA sequences(18S coding sequence plus spacer)as a probe along with an enzyme—1inked colour reaction to hybridize to MM106 chromosomes. The position of the nucleolar organiser regions, the site of ribosomal RNA transcription[14, 15] was determined by silver staining to provide independent verification of the rDNA probe results. The number of active ribosomal gene loci can also be confirmed by counting nucleoli (sites of ribosome biogenesis)in interphase nuclei[13, 14, 16-18].

MAI'ERIALS AND METHODS

Plant material

Actively growing root tips were harvested from glasshouse-grown cuttings of the apple rootstock 'MM106' 'Northern Spy' (Malus x domestica Borkh.) x Malling I-derived from *M. paradisia*[19],

Preparation of mitotic chromosome spreads and Giemsa staining

Root tips were pretreated with a saturated paradichlorobenzene solution for 5-6 h at room temperature, rinsed in distilled water f or 5 min and fixed in freshly prepared acidic acid / ethanol (1:3) at 4℃ overnight. Cell walls were hydrolysed by incubation in a solution of 2%cellulase and 2%pectinase(Calbiochem-Behring)at pH 4. 5 for 90-120 min at 37℃ . Roots were washed 2-3 min in distilled water and transferred to 45%acetic acid before scraping out, macerating. and squashing the meristematic cells. Preparations were frozen in liquid air, coverslips were removed and slides were immersed in 95%ethanol for 30-60 min before air drying and then stored at-70℃. Following Giemsa staining[20], mitotic spreads with cleanly separated chromosomes were identified, photographed under an on objective and karyotypes prepared (Fig 1A).

In situ hybridisation

The rDNA used as a probe (a gift from Dr. C J Simon)was a 6kb rDNA fragment from genomic DNA isolated from the crab apple 'Whire Angel' cloned into pUCl3. It included an 18S coding region and an intergenic spacer[21]. The fragment was excised with Eco RI and then labelled with digoxygenin-dUTP by random priming according to the manufacturer's instructions (Boehringer Mannheim GmBh).

Prior to hybridisation, metaphase chromosome preparations were destained in acetic acid ethanol (1:3) for approximately 5-10 mih, transferred to 95% ethanol, and air dried. The slides were treated with DNase-free RNase f(100μ g/ml in 2 x SSC) for 60 min at 37℃, rinsed briefly in 2 x SSC at room temperature, and incubated for 30 min in 2 x SSC at 70℃ before dehydration through 70%,

95%and 100%ethano1. Slides were then air dried.

Twenty-five μl of hybridisation mixture (20-40 ng of digoxygenin labelled probe in 6 x SSC, 45%formamide and 5 x Denhardt's solution) was placed on each side, covered with a siliconized coverslip (22 x 22 mm) and sealed with a fast-drying glue. Denaturation was performed by placing slides on a hot plate at 84.5-85 ℃ (fmonitored by a thermopile) for 7-10 min and quenching was carried out on ice for 5 min. Hybridization was performed overnight in a humidity chamber at 40℃. After removing the coverslips, slides were washed thoroughly three times with 2 x SSC at room temperature for 5 min, with PBS and 0.1%Triton X-100 for 3 rain and then again three times in PBS.

Controls included either a preparation pretreated with 50 μg/ml DNase for 30 min at 37℃ or a normal preparation carried through the hybridization procedure but without a probe.

Detection of sites of hybridization of the digoxygenin-labelled probe with an antibody-enzyme conjugate (anti-digoxygenin-alkaline phosphatase) and its visualisation by an enzyme-linked colour reaction (substrates BCIP/NBT) was according to the manufacturer's instructions (Boehringer Mannheim GmbH). The preparations were photographed and the sites of hybridization assigned to specific chromosomes according to the Giemsa karyotype.

Silver staining of nucleolar organiser regions(NORs)

Giemsa-stained metaphase chromosome preparations were first destained, and then re-stained with silver nitrate according to either one of two equally successful methods. Following the procedure of Lacadena *et a1.*[17], 3-4 drops of freshly prepared 0.7% (w/v) aqueous silver nitrate(adjusted to pH 3.0 with formic acid) on chromosome preparations, spread on slildes covered with coverslips and incubated overnight in a humidity chamber at 50℃. The other technique involved the one-step colloidal silver method as described by Howell and Black[22]. Sites of intense black staining (NORs) were assigned to chromosomes. Sites of brown or diffuse staining were non-specific in nature and were ignored.

Chromosome measurements

Measurements were made from photographs of eighteen metaphase spreads for all three types of staining and relative length, arm ratio, were calculated according to Macgregor and Varley[20].

Detection of nucleoli

Interphase cells from root tips were stained by the aqueous silver nitrate/colloidal developer method of Howell and Black[22].

RESULTS and DISCUSSION

Analysis of eighteen metaphase spreads stained by Giemsa, silver staining, and in situ hybridization confirmed that 'MM106' is a diploid (2n=34) and that its chromosomes can be arranged in seventeen pairs (Figs 1 and 2) [23].The measurements of relative chromosome length, and arm ratio indicated that a11 the chromosomes are submetacentric (Tab 1). Their size ranged from 1.0-2.7 μm, which is slightly larger than reported earlier (0.5-1.0 μm) by Lespinasse et *a1.*[23]. Secondary constrictions which have been associated with plant NORs in some studies [4, 5, 10, 16, 24] were not observed.

Hybridization of the rDNA sequence was detected at a 90% frequency(over ten preparations) as patches of dark purplish-brown precipitate at the end of the short arms of chromosomes 6 and 14 (Fig 1b), while control preparations(either DNase

pretreatment of chromosomes or hybridization without a probe) failed to give such a colour reaction.

Nucleolar organiser regions stained with silver were visible as tight black spots whereas non-specific staining was yellowish brown and tended to be more diffuse. The ten preparations examined indicated the presence of NORs at a frequency of 70% for chromosome 6 and 90%for chromosome 14. NORs were present at the end of the short arms for both chromosome pairs. These results confirm the localization of ribosomal RNA genes detected with in situ hybridisation by the rDNA probe (Fig 2) since the acidic silver staining of ribonucleic protein accumulating around active NORs in interphase and remaining at mitosis is an indication of gene activity at rDNA sites during the preceding interphase[14].

Tab 1.Chromosome measurements for apple rootstock 'MM106' (mean of eighteen metaphase preparations with results pooled for the three methods of staining).

Chromosome number	Relative length	Arm ratio
1	8.70	2.14
2	7.77	1.77
3	7.34	2.20
4	7.01	2.24
5	6.76	2.15
6	6.53	1.80
7	6.30	2.06
8	5.97	2.04
9	5.77	2.03
10	4.43	1.94
11	5.30	1.85
12	5.07	2.22
13	4.83	1.76
14	4.67	1.66
15	4.56	1.76
16	4.23	1.71
17	3.77	1.59

All chromosomes are submetacentric.

When silver stained interphase preparations were screened to determine the maximum number of nucleoli and hence number of active rDNA loci，most interphase nuclei were found to contain one large nucleolus formed by complete fusion of nucleoli during the cell cycle，but few contained two, three, or the primary number of four[25], indicating that all four rDNA loci can be active as reported for AraNdopsis[13]. The nucleoli were of two size classes (Fig 3), reflecting differences in amount or rate of transcription at the rDNA loci on chromosome pairs 6 and 14, or difierent rates of assembly or export of ribosomes[26].

Onr finding that rDNA genes in *Malus* are localized on two pairs of chromosomes is consistent with earlier *in situ* hybridization for the diploid angiosperm species including *A11ium* cepa，A. *fistulogum*, *AraNdopsis thaliana*，*Pisum sativum*，and *Hordeum vulgare*. In contrast in Secale cereale hybridization only one pair of chromosomes has been reported [4, 5, 13, 24].

CONCLUSIONS

The combination of *in situ* hybridization of metaphase preparations with a homologous rDNA probe with acidic silver staining of NORs and nucleoli has enabled

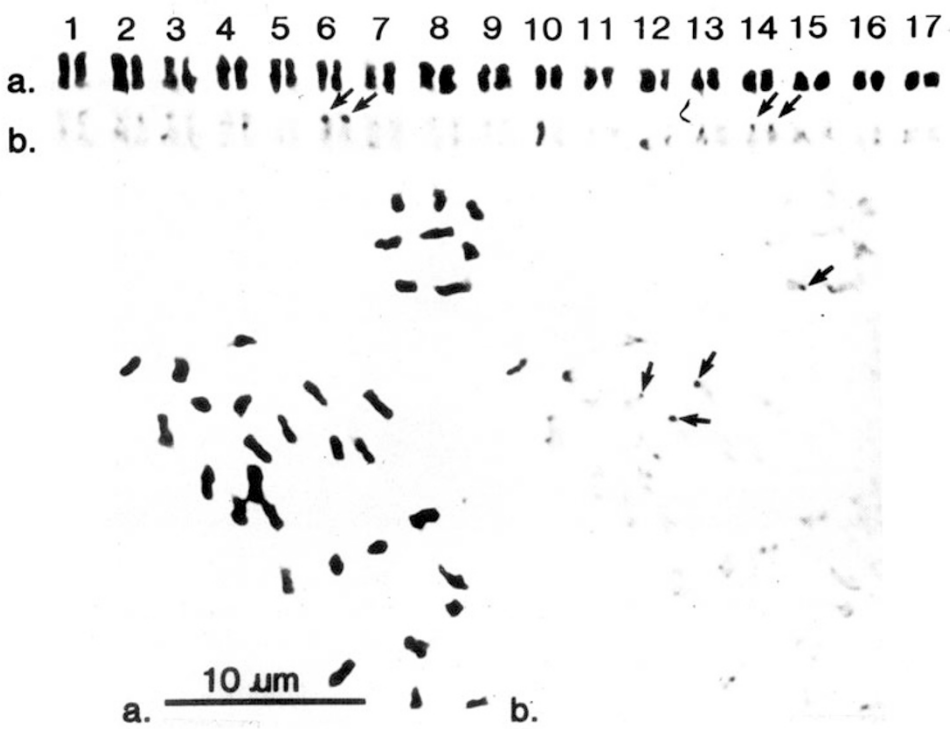

Fig 1. Root tip metaphase chromosome spread of apple rootstock 'MM106' with karyotype.
a. Giemsa staining.
b. *In situ* hybridization with an 18S plus spacer rDNA probe detected with the alkaline phosphatase-linked immunoassay. Sites are indicated by arrows.

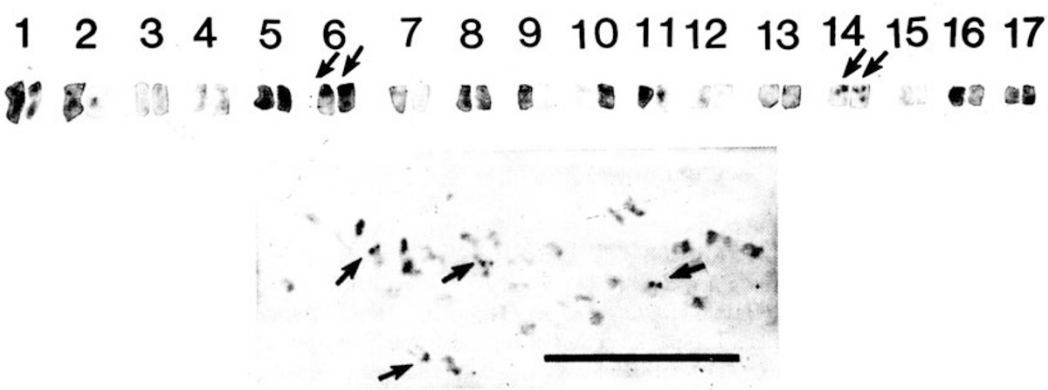

Fig 2. Silver staining of root tip metaphase chromosomes of apple rootstock 'MM106'. Ar-. rows indicate NORs on spread and karyotype. Bar: 10 μ m

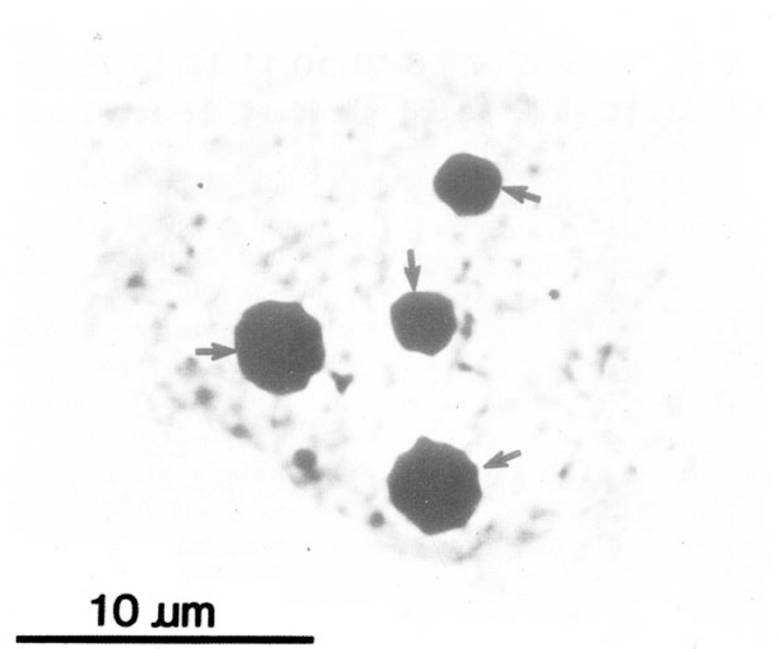

Fig 3 . Silver-stained interphase nucleus of apple rootstock 'MM106' showing 4 nucleoli.

the assignment of rDNA genes to the telomeric regions of the short arms of chromosomes 6 and 14 in the apple rootstock 'MM106'. The digoxygenin labelling of the probe coupled with an antibody-alkaline phosphatase colour detection system proved to be a sensitive, reliable and rapid method for the identification of sites of hybridization to small chromosomes (1.0-2.7 μm) of this woody perennial.

Future studies for *in sjtu* hybridization to apple chromosomes will ultimately relate information from linkage maps to the physical chromosomes.

Acknowledgement

This research was funded by the New Zealand Foundation for Research, Science and Technology, Contract Number C06209.

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Received l-12-1 994 *Accepted 2-3-1995.*