Cell Research(1995),**5**,243–253

Physical mapping of three fruit ripening genes: Endopolygalacturonase, ACC oxidase and ACC synthase from apple (*Malus x domestica*) in an apple rootstock A106 (*Malus sieboldii*)

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ABSTRACT

The apple rootstock, A106 (Malus sieboldii), had 17 bivalents in pollen mother cells at meiotic metaphase 1, and 17 chromosomes in a haploid pollen cell. Karyotypes were prepared from root-tip cells with 2n = 34 chromosomes. Seven out of 82 karyotypes (8.5%) showed one pair of satellites at the end of the short arm of chromosome 3. C-bands were shown on 6 pairs of chromosomes 2, 4, 6, 8, 14, and 16 near the telomeric regions of short arms. Probes for three ripening-related genes from Malus x domestica: endopolygalacturonase (EPG, 0.6 kb), ACC oxidase (1.2 kb), and ACC synthase (2 kb) were hybridized in situ to metaphase chromosomes of A106. Hybridization sites for the EPG gene were observed on the long arm of chromosome 14 in 15 out of 16 replicate spreads and proximal to the centromere of chromosomes 6 and 11. For the ACC oxidase gene, hybridization sites were observed in the telomeric region of the short arm of chromosomes 5 and 11 in 87% and 81% of 16 spreads respectively, proximal to the centromere of chromosome 1 in 81% of the spreads, and on the long arm of chromosome 13 in 50% of the spreads.

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Twenty five spreads were studied for tile ACC synthase gene and hybridization sites were observed in the telomeric region of the short arm of chromosome 12 in 96% of the spreads, chromosomes 9 and 10 in 76% of the spreads, and chromosome 17 in 56% of the spreads.

Key words: *Malus, chromosome, in situ hybridization C-banding, karyotype.*

INTRODUCTION

In situ hybridization of DNA probes to chromosomes using non-radioactive detection systems has been used in a number of crops including wheat [1], pea[2-4], and apple[5]. The physical mapping of genes in apple is part of an international effort to map the apple genome[6-8].

This paper describes the karyotype of an apple rootstock cultivar A106 (*Malus sieboldii*) which has been characterised by analysis of the meiotic metaphase I chromosomes, somatic mitoses and C-banding pattern. Probes for three ripening-related genes (endopolygalacturonase (EPG), ACC oxidase, and ACC synthase from *Malus x domestica*) were hybridized *in situ* to the metaphase chromosomes spreads of A106 in order to locate the physical position of the corresponding genes on an apple karyotype.

MATERIALS AND METHODS

Plant material

Young branches of the apple rootstock A106 (Malus sieboldii) were cut, planted in pots and kept in a green house for several weeks before being transfered outdoors to initiate the growth of young roots for use in mitotic chromosome spreads.

Cytogenic studies

Meiotic analysis

Flower-buds of A106 were harvested just as they emerged from the very young leaf sheath in September for use in the preparation of meiotic chromosome spreads. Meiotic chromosome counts were performed by following the procedure of Lespinasse et al[9]. Young flower buds were fixed in a Carnoy solution (6:3:1 absolute ethanol:chloroform:glacial acetic acid) mordanted with a few drops of ferric chloride solution and stored at 4 ℃ overnight or a few days before use. Meiotic spreads were prepared by squashing pollen mother cells in a drop of Acetocarmine staining solution. Chromosome spreads of cells in metaphase 1 were examined for chromosome structure and the presence of bivalents.

Preparation of mitotic chromosome spreads

Mitotic chromosome spreads from young root-tips of A106 were prepared according to Zhu and Gardiner[5]. Giemsa staining was carried out according to the method of Schweizer[10] and well separated mitotic chromosome spreads were recorded and photographed under an oil immersion 100

x objective using a Zeiss Carol microscope. These chromosome spreads were used for C-banding and the in situ hybridization studies as described below.

Chromosome C-banding

Mitotic slides were aged at room temperature for 3-10 d. The protocol for C-banding was carried out by a modified procedure derived from Summer[11], Seal and Bennet[12], Kakeda et al[13], Heneen and Brismar[14] and MacGregor and Varley[15]. The protocol was as follows. The aged slides were denatured in a fleshly prepared 5% $Ba(OH)_2$ solution at 60℃ for 30 min. Following washing in distilled water at room temperature, slides were renatured in 2 × SSC (SSC: 0.15*M* sodium chloride and 0.015*M* sodium citrate) at 60℃ for 2 h. One of two alternative procedures was then followed for staining. In the first procedure slides were rinsed in distilled water, and then stained in 5% Wright's solution[12] for 1-2 h at room temperature. In the second procedure slides were immediately dipped in 0.02% trypsin (Difco Bacto-trysin) for 30 min, rinsed in distilled water for 2 min at room temperature and stained in 5% Wright's solution. If the C-bands were not sharp enough, slides were destained in 95% ethanol for 5 min, dried in air, retreated with trypsin for 20-30 min at room temperature, rinsed in distilled water and restained in 5% Wright solution or Giemsa solution.

Probe preparation

Primers for a Prunus endopolygalacturonase were obtained from G. King, Horticulture Research International, Wellesbourne, England. Sequences were:

GK5 5'-ACT TGT GGA CCA GGC CAT GGA

GK6 5'-CAC ATT TTT CAC TTG AAC TGC.

These primers were used to amplify DNA from *Malus x domestica* cv. 'Scijoy' with reaction conditions similar to those for probe synthesis (see below), but lacking added $MgCl_2$ and with the addition of 1.5% formamide. The products were electrophoresed in a 2% agarose gel and the major product of 0.6 kb was excised and purified using Prep-a-Gene (Biorad, Hercules, Ca, USA). An aliquot of this was employed as template for the synthesis of biotin labelled probe. The 1.2 kb ACC oxidase clone[16] and the 2 kb ACC synthase clone[17] were isolated from a cDNA library prepared in pSPORT from polyA+ RNA extracted from ripe apple cortical tissue.

Biotin-labelled probe was prepared by the method of Emanuel[18] using 40 cycles of PCR amplication reaction in a thermal cycler (Perkin Elmer Cetus). The first cycle consisted of 4 min at 94℃, 1 min at 55℃, and 2 min at 72℃. The second and subsequent cycles were 1 min at 94℃, 1 min at 55℃, and 2 min at 72℃, with 8 min extension time at 72℃ after the final cycle. An additional 5 units of Taq polymerase (Stratagene, La Jolla, Ca, USA) was added after the 20th cycle.

Each PCR reaction (100 μ1) contained 5 units of Taq polymerase (Stratagene), 5-20 ng DNA template, 5:1 of dNTP (containing 2 mM dCTP, dATP, dGTP; 1.34 m*M* dTTP; 0. 66 m*M* biotin-16-dUTP (Boehringer)), 6.3 ng SP6 promoter primer and 6.7 ng T7 promoter primer, 4 μl of 12.5 m*M* of $MgCl_2$, 10:1 of 10 x PCR reaction buffer as supplied by the manufacturer (100 m*M* Tris-HCl pH 8.8, 500 m*M* KCl, 1.5 m*M* $MgCl_2$,, 0.1% gelatin) and H_2O to a final volume of 100μl . Each of the ripening-related genes was labelled individually and the probes were purified using Prep-a-Gene (Biorad) after checking by agarose gel electrophoresis that a single band of the correct size had been synthesized. Biotin label quantitation using Dot Blot was carried out according to the manufacturer's instructions ("BluGENE", Life Technologies, Bethesda, Md, USA).

In situ hybridization

Prior to hybridizations, mitotic slides prepared from young root tips were processed through a series of treatments. Slides (stored at -70℃) were refixed in 1:3 acetic acid: 95% ethanol for 1 h, transfered to 95% ethanol and then to absolute ethanol (5 min each step) and dried in air for 5-10 min. Slides were treated with DNase-free RNase (100 μg/ml in 2 × SSC) for 60 min at 37℃, rinsed briefly in 2 × SSC at room temperature, and then treated with 50 μl of 40 mg/ml

proteinase K (Sigma) in PBS buffer, covered with a coverslip at 37℃ for 20 min, refixed in a freshly prepared 4% paraformaldehyde in PBS buffer at room temperature for 1 min, dehydrated by serial ethanol immersions (70%, 90%, and 100% ethanol, 3 min each) and dried in air for 5-10 min. The in situ hybridization of three biotinylated cDNA probes (EPG, ACC oxidase and ACC synthase genes) were performed according to manufacturer's instructions (BRL, Life Technologies). The hybridization solution contained 50% formamide, 2 × SSC, 10% dextran sulphate, 1 x Denhardts solution (BRL Life Technologies) and 10 μg/ml of biotinylated PCR product. Twenty to thirty microliters of this hybridization mixture was applied onto each slide. Each slide was covered with a siliconised coverslip, placed onto a preheated plate at 100℃ for 5 min, and then immediately quenched on ice for 3-5 min. Following sealing of the coverslip onto the slide with rubber cement, the hybridization was performed at 42℃ overnight. The coverslips were removed carefully and slides were washed 4 times in 0.2 × SSC at 42℃ (3-4 min) and 3 times in 0.2 × SSC at room temperature (5 min). The biotinylated hybridization signal was detected using the streptavidin conjugated alkaline phosphatase in conjugate dilution buffer (100 mM Tris-HCl, 150 m*M* $MgCl_2$, 10 mg/ml bovine serum albumin) according to manufacturer's instructions (BRL, Life Technologies). The alkaline phosphatase was visualized by incubating the slide at 37℃ in a chromogenic substrate solution. This substrate solution consists of 200 μ1 of nitro blue tetrazolium (75 mg/ml dissolved in 70% (v/v) dimethylformamide) and 166 μ1 of 4-bromo-5-chloro-3-indolylphospate (50 mg/ml dissolved in 100% dimethylformamide) in 50 ml of alkaline-substrate buffer (100 m*M* Tris base, 150 mM Sodium Chloride, 50 mM Magnesium Chloride at pH 9.5). Levamisole (10 mg) was added to inhibit residual alkaline phosphatase activity. The alkaline phosphatase enzyme activity associated with the probe forms as localized purple precipitate. Colour development was assessed under a phase contrast microscope until purple precipitates could be detected on chromosomes (within 2 h). Negative control slides without the probe were prepared in each in situ hybridization experiment.

Hybridization signals which appeared as symmetrical spots on both chromatids of at least one of the two homologous chromosome were recorded as positive. Signals which occurred in fewer than 50% of the spreads were not recorded. Positive control slides (paraffin-embedded sections of adenovirus type 2-infected Hela cells) were hybridized with positive probe and developed using the same procedures as described for samples to confirm the efficiency of the procedure employed. Photographs for meiotic spreads, mitotic spreads, C-banding and in situ hybridization were taken with Kodak Technical Pan 2415 film, under an oil immersion (100 × objective) using a Zeiss Carol microscope.

RESULTS

Meiotic chromosome counts

Pollen mother cells of A106 showed 17 bivalents in meiotic metaphase 1 (Fig 1A) and a haploid pollen cell contained 17 chromosomes (Fig 1B) confirming that A106 is diploid with a chromosome complement 2n=34.

Mitotic chromosome spreads and C-banding

Examination of mitotic chromosomes from root tips stained by conventional methods using a 5% Giemsa solution demonstrated the existance of morphologically similar homologues (Fig 2A). In seven out of 82 karyotypes studied, a pair of satellites were observed at the end of the short arm of chromosome 3 (Fig 2B). In order to identify the homologous pairs of chromosomes, C-banding in 11 karyotypes was studied in detail. Differential staining of C-bands was observed near the telomeric region of the short arm of chromosomes 2, 4 and 14 at a frequency of 72%,

chromosomes 8 at 54% and chromosome 6 and 16 at 45% (Fig 3).

The well separated chromosome-spreads were photographed. Measurements of the length of the short arm (S) and the long arm (L) were taken from enlarged photographs of 16 intact cells. The mean lengths and arm ratios (L/S) are presented in Tab 1. Chromosome size ranged from 1.18 μm to 2.82 μm, and the arm ratio (1.7 to 3). Chromosomes in A106 were all submetacentric based on the criteria of Levan et al[l 9].

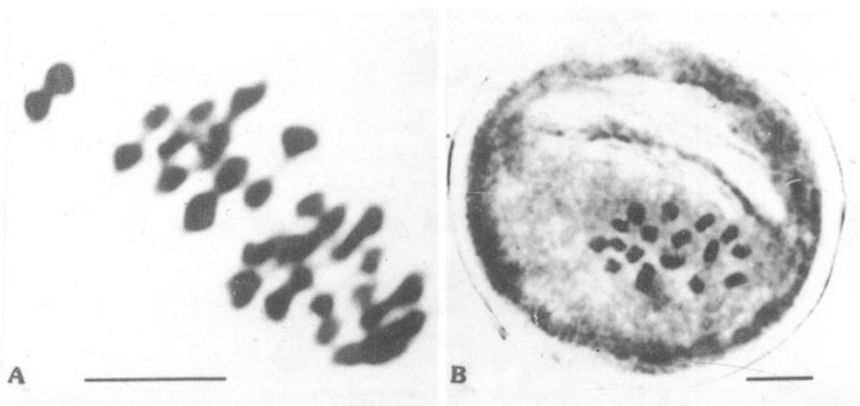

Fig 1. Acetocarmine stained pollen mother cells
(A) 17 bivalents in a diploid pollen mother cell at meiotic metaphase 1.
(B) 17 chromosomes in a haploid pollen cell of A106 (Malus sieboldii).

In situ hybridization analysis

Hybridization sites for EPG were observed on the long arm of chromosome 14 in all but one of the 16 spreads studied and were proximal to the centromere on chromosomes 6 and 11 in 75% of the spreads, (Fig 4). An additional 16 spreads studied for ACC oxidase revealed hybridization sites in the telomeric region of the short arm of chromosomes 5 and 11 in 87% and 81% of the spreads respectively, proximal to the centromere of chromosome 1 in 81% of the spreads, and on the long arm of chromosome 13 in 50% of the spreads (Fig 5). Hybridization sites were observed for ACC synthase in the telomeric region of the short arm of chromosome 12 in 96% of the spreads studied, chromosomes 9 and 10 in 76% of the spreads, and chromosome 17 in 56% of the spreads (Fig 6). Negative control spreads showed no signal or colour reaction, and positive control slides showed strong purple precipitates at the site of Hela cells infected with adenovirus type 2.

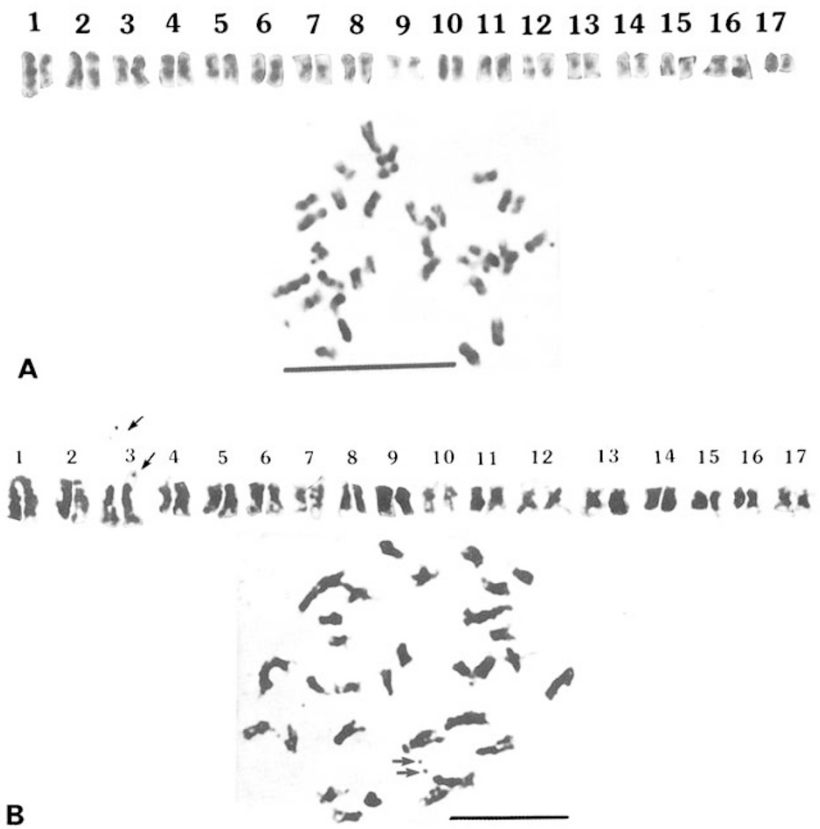

Fig 2. Giemsa stained karyotype of root-tips of A106 (*Malus sieboldii*)
(A) 17 homologous pairs of chromosomes. Bar = 10 μm
(B) One pair of satellites showing at the end of the short arm of chromosome 3 (at late prophase). Bar = 10 μm

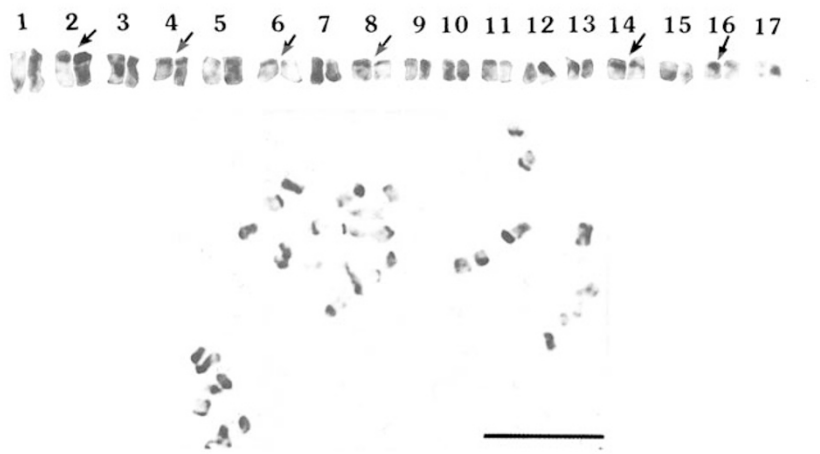

Fig 3. C-banding karyotype of root-tips of A106 (*Malus sieboldii*). Bar = 10 μm

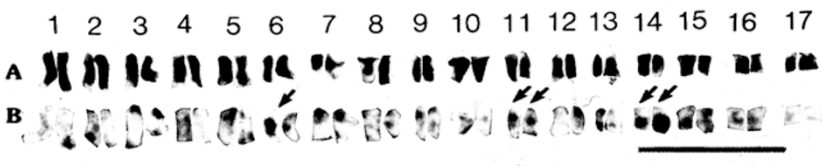

Fig 4. *In situ* hybridization with biotin labelled 0.6 kb cDNA of endopolygalacturonase (EPG).
(A) Giemsa stained karyotypes.
(B) *In situ* hybridization sites of EPG. Bar = 10 μm.

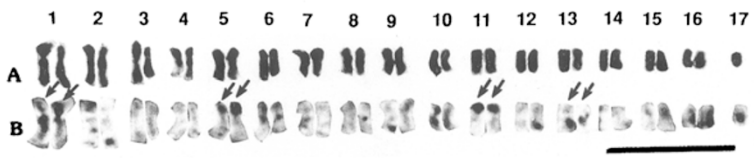

Fig 5. *In situ* hybridization with biotin labelled 1182 bp cDNA of ACC exidase.
(A) Giemsa stained karyotypes.
(B) *In situ* hybridization sites of ACC oxidase. Bar = 10 μm.

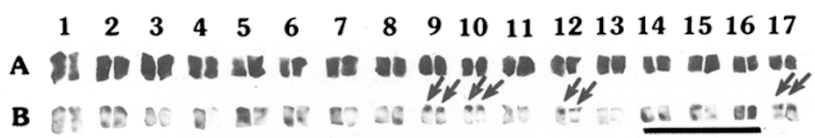

Fig 6. *In situ* hybridizatioil with biotin labelled 2 kb cDNA of ACC synthase.
(A) Giemsa stained karyotype.
(B) *In situ* hybridization situs of ACC synthase. Bar = 10 μm.

DISCUSSION

It is reported that Malus sieboldii has a diploid chromosome number 2x = 34, 3x = 51, 4x = 68 and 5x = 85 [20]. In New Zealand, analysis of the apple rootstock *Malus sieboldii* A106 demonstrated that it has a haploid chromosome number of 17 and a diploid chromosome number 2n=34 based on chromosome counts of meiotic metaphase pollen mother cells, the pollen cell, and mitotic chromosome spreads

of root-cells. A small pair of satellites was sometimes observed on one or both of the homologues of chromosome 3. The low frequency appearance of satellites is probably caused by the poor quality of spreads and the relatively small size of the apple chromosome (1.18-2.82 μm). Late prophase and early metaphase root-tip cells with the least cytoplasm produced the best mitotic chromosome spreads for karyotyping.

Tab 1. Mitotic measurements for apple rootstock 'A106' (*Malus sieboldii*)
(Mean of sixteen somatic chromosomes)

Chromosome number	Relative length	Arm ratio
1	9.21	2.04
2	7.93	2.15
3	7.40	2.06
4	6.97	2.14
5	6.63	2.17
6	6.26	1.86
7	6.09	1.75
8	5.83	2.14
9	5.58	1.83
10	5.45	1.97
11	5.26	2.01
12	5.09	1.96
13	4.91	1.98
14	4.68	1.84
15	4.49	1.98
16	4.36	1.96
17	3.87	1.81

All chromosomes are submetacentric according to the criteria of Levan et al (1964)

The differential staining technique of C-banding was used for the first time in apple to identify pairs of homologous chromosomes. C-bands at the terminal regions of the short arm of chromosomes 2, 4, 14 were apparent in more than 70% of the spreads and at the terminal region of chromosome 8 in more than 50% of the spreads. C-bands were apparent in fewer than 50% of spreads at the terminal regions of the short arm of chromosomes 6, 7, and 16. Centromeric C-bands were detected during prophase on chromosomes 7, 10 and 13 in only 2 of 16 karyotypes, and no other C-bands were detected in this study. C-bands are thought to represent highly repeated DNA sequence or heterochromatin regions which can be used in some chromosome karyotypes as markers to identify individual chromosomes. This information can then be used for in situ hybrization studies. In apple it is apparent that C-banding, on its own, is not sufficient to distinguish all 17 homologues or for detailed localization of genes on chromosomes, but it may be useful in distinguishing certain chromosomes which are similar in size and arm ratio (e.g. chromosomes 4 and 5).

Tab 2. EPG, ACC oxidase and ACC synthase hybridization sites on A106 chromosomes

Chromosome number	Gene location		
	EPG (16 spreads)	ACC Oxidase (16)	ACC synthase (25)
1		Proximal (81%)	
2			
3			
4			
5		NT(S) (87%)	
6	Proximal (75%)		
7			
8			
9			NT (S) (76%)
10			NT (S) (76%)
11	Proximal (75%)	NT (S) (81%)	
12			NT (S) (96%)
13		L (50%)	
14	Proximal L (> 94%)		
15			
16			
17			NT (S) (56%)

EPG: endopolygalacturonase
Proximal: Proximal to the centromere NT: Near the telomeric region
L: Long arm S: Short arm

The gene expression and regulation of the enzymes EPG, ACC oxidase and ACC synthase have been shown to be important in the ripening of a number of fruit[21]. In apple, Southern analysis has produced evidence that these enzymes are encoded by multigene families[16, 17, 22, 23]. Through in situ hybridization, the present study indicates that genes homologous to EPG, ACC oxidase and ACC synthase are located in different regions on a total of 10 pairs of chromosomes. Genes homologous to EPG were located on three different chromosomes, and those homologous to ACC oxidase and ACC synthase were each located on four different chromosomes (Tab 2). Location of different members of multigene families on separate chromosomes has also been demonstrated for the ACC synthase multigene family in tomato[24]. The results in the present work demonstrate that a Streptavidin conjugated alkaline phosphatase detection system can be used to locate genes on an apple karyotype by in situ hybridization.

Other methods of in situ hybridization have used fluorescein as a non-isotopic reporter system to detect hybridization signals in plants such as wheat[l, 25], Allium[26], soybean[27] sugar beet[28] and Solanum brevidens[29]. Fluorescence in situ hybridization (FITC) and chromosome banding in humans were successfully integrated into a single procedure[30]. DNA sequences can be directly and precisely mapped to a specific chromosome area by combining these techniques[l, 31]. Fluoresence-banding using dyes such as DAPI has been recently reported in citrus

by Guerra[32] who demonstrated the existence of heterochromatin at the terminal region of long arms. A different banding pattern was apparent in each species. A similar approach using fluorescent dyes might be possible in apple chromosomes and any markers revealed would be useful for chromosome identification. An alternative approach that could be taken is to use the simultaneous visualization of in situ hybridization signals for single genes and a suitable method for producing banding patterns with fluorescent dyes or fluorescently labelled repeated DNA. This might enable more precise physical mapping of genes in apple and increase the efficiency of map construction.

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Received 29-9-1995. Revised 20-11-1995. Accepted 6-12-1995