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FISH to meiotic pachytene chromosomes of tomato locates the root-knot nematode resistance gene *Mi-1* and the acid phosphatase gene *Aps-1* near the junction of euchromatin and pericentromeric heterochromatin of chromosome arms 6S and 6L, respectively

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Abstract The root-knot nematode resistance gene *Mi-1* in tomato has long been thought to be located in the pericentromeric heterochromatin region of the long arm of chromosome 6 because of its very tight genetic linkage (approx. 1 cM) to the markers *Aps-1* (Acid phosphatase 1) and *yv* (yellow virescent). Using Mi-BAC clones and an Aps-1 YAC clone in fluorescence in situ hybridisation (FISH) to pachytene chromosomes we now provide direct physical evidence showing that *Mi-1* is located at the border of the euchromatin and heterochromatin regions in the short arm (6S) and *Aps-1* in the pericentromeric heterochromatin of the long arm (6L) close to the euchromatin. Taking into account both the estimated DNA content of hetero- and euchromatin regions and the compactness of the tomato chromosomes at pachytene (2 Mb/μm), our data suggest that *Mi-1* and *Aps-1* are at least 40 Mb apart, a base pair-to-centiMorgan relationship that is more than 50-fold higher than the average value of 750 kb/cM of the tomato genome. An integrated cyto-genetic-molecular map of chromosome 6 is presented that provides a framework for physical mapping.

Key words Fluorescence in situ hybridisation FISH · Pachytene chromosomes · Nematode resistance gene · *Mi* · Tomato

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Introduction

Root-knot nematodes (*Meloidogyne* spp) cause serious damage to many crops throughout the world. The molecular cloning and tailoring of plant genes conferring resistance against nematodes have thus been a major challenge to molecular biologists and breeders from both a fundamental and applied perspective (see review of Williamson and Hussey 1996; Liharska and Williamson 1997). Recently, the molecular cloning of a tomato nematode resistance gene (*Mi*) has been accomplished (Kaloshian et al. 1998; Milligan et al. 1998). Thus, more than 50 years after the identification of host-encoded nematode resistance (Baily 1941), the stage has been set for an in-depth analysis of the nematode-tomato interaction and subsequent application of the cloned resistance gene in practical breeding.

In the early forties, a high level of resistance to root-knot nematodes was found to occur in *L. peruvianum*, a wild relative of the cultivated tomato *L. esculentum* (Bailey 1941). This resistance was successfully introgressed into *L. esculentum* (Smith 1944) and further accommodated to commercial lines by repeated backcrossing (Frazier and Dennett 1949; Gilbert and McGuire 1956). The introgressed trait was referred to as *Mi* from the first letters of the nematode species *M. incognita* (Gilbert and McGuire 1956). Remarkably, *Mi* was also found to be effective against two other major nematode species, *M. javanica* and *M. arenaria* (Barham and Winstead 1957). Genetic studies revealed that the resistance governed by *Mi* is dominant and segregates as a single major locus (Gilbert and McGuire 1956) that has been mapped on chromosome 6 in tight linkage (approx. 1 cM) to both the leaf colour marker *yv* (yellow virescent) (Gilbert 1958) and the acid phosphatase-1^1 (*Aps-*1^1) locus (Rick and Fobes 1974). This linkage relationship has been extensively exploited for indirect selection of nematode resistane in tomato breeding programmes.

On the basis of deletion mapping (Khush and Rick 1968), *yv* was assigned to the pericentromeric heterochromatin region of the long arm of chromosome 6 at a genetic distance of about 7 cM from another leaf morphology marker, *tl* (thiamineless), on the short arm. Because of its very tight linkage to both the *yv* locus and the *Aps-1* locus, *Mi* has been placed on the long arm near *yv* in original versions of the classical genetic maps of tomato chromosome 6 (Khush and Rick 1968; Tanksley et al. 1992; see also Weide et al. 1993). However, evidence based on physical data from deletion mapping experiments using molecular markers tightly linked to *Mi* (van Wordragen et al. 1994) shows *Mi* to be located on the short arm rather than on the long arm. This has been further substantiated by means of a detailed molecular mapping of numerous irradiation-induced deletions that involved both the *Mi* gene and the morpholigical marker *tl* (Liharska et al. 1997). These studies have thus provided a precise location of *Mi* on the integrated molecular/classical linkage map (see also van Wordragen et al. 1996).

In the investigation presented here we completed the map integration by establishing the chromosomal, cytogenetic map position of *Mi* and *Aps-1* as revealed by fluorescence in situ hybridisation (FISH) to pachytene chromosomes using as probes BAC and YAC clones derived from the respective regions. FISH to extended DNA fibres was used as an internal control for the molecular position of the BACs on the chromosomal DNA.

Materials and methods

Tomato DNA clones

Two BAC clones, BAC1 and BAC3 (see Milligan et al. 1998; see also Fig. 1c), were selected from a BAC library made from a yeast strain carrying YAC2/1256 which includes the entire region to which *Mi* has been localised (Kaloshian et al. 1998). The BAC DNAs were isolated by an alkaline lysate method (Woo et al. 1994) and further purified by the GlassMAX DNA isolation reagent system (Life Technologies). The tomato DNA inserts were released from the BAC clones by *Not*I digestion, and their molecular sizes were estimated by pulsed field gel electrophoresis (PFGE) for 20 h at an initial pulsed time of 1 s and a final pulsed time of 10 s at voltage of 6.0 V/cm. An Aps-1 YAC clone (50 kb) isolated from a tomato YAC library (Bonnema et al. 1996) was purified by cutting the YAC-containing band from a preparative gel after pulse field gel electrophoresis as described by van Wordragen et al. (1998).

Probe labelling

BAC1 DNA was labelled with digoxigenin-11-dUTP, and BAC3 DNA and Aps-1 YAC DNA with biotin-16-dUTP, using standard nick translation according to the instructions of the manufacturer (Boehringer Mannheim, Germany).

Plant materials and microscopy preparation

Lycopersicon esculentum cv 'VFNT cherry', which carries an introgressed region of *L. peruvianum* at chromosome 6 containing the nematode resistance gene *Mi* (Ho et al. 1992; Liharska et al. 1997), was used for FISH mapping. Pachytene chromosome preparations were made from young anthers according to the protocol of Zhong et al. (1996a). Preparations of extended genomic DNA fibres were made from young leaf material (Fransz et al. 1996, 1998; Zhong et al. 1996b).

Fluorescence in situ hybridisation (FISH)

Two-coloured FISH of the BAC clones and the YAC clone to pachytene chromosomes and extended DNA fibres was performed according to the protocol of Zhong et al. (1996b). Digoxigenin-labelled BAC1 probe was detected by anti-digoxigenin-FITC showing green fluorescent hybridisation signals, while the biotin-labelled BAC3 and the *Aps-1* YAC probes were detected by avidin-Texas Red showing red fluorescence. Chromosomes were counterstained with DAPI. FISH results were photographed on 400 ASA colour negative films using a Zeiss fluorescence microscope equipped with epifluorescence illumination and filter 25 with separated excitation filters for observation of DAPI, FITC and Texas Red. Two- or three-coloured FISH signals were simultaneously recorded on one photograph by double or triple exposure. The pictures were converted to digital images by scanning the negative films. The images shown in the figures were contrast-enhanced using Photoshop 3.0 image processing software. Thirty images of the BAC signals on extended DNA fibres without overlap with other DNA fibres were selected for analysis of physical mapping data. Ten of the fluorescence profiles (in Fig. 1b) were aligned based on positions of overlapping parts.

Results

Molecular characterisation of BAC1 and BAC3

In order to establish the extent of overlap between BAC1 (57 kb) and BAC3 (50 kb), both of which correspond to the *Mi*-region (Milligan et al. 1998) and to establish that each BAC represented a contiguous stretch of DNA (i.e., was not a chimaera or other cloning artefact), the BACs were subjected to FISH to extended genomic DNA fibres. Previous studies have shown that Fibre-FISH provides a convenient and powerful alternative to restriction mapping for such an analysis (Fransz et al. 1996, 1998). Hybridisation signals of digoxigenin-dUTP-labelled BAC1 and biotin-dUTP-labelled BAC3 were detected using anti-digoxigenin-FITC and avidin-Texas Red, respectively, and appeared as green and red fluorescent tracks (Fig. 1a, b). The green fluorescent signal of BAC1 was directly flanked by the red signals of BAC3 with a small yellow region in between, indicating the partial overlap of the two BACs.

Thirty representative hybridisation signals on fibres that did not overlap with other fibres were selected for further analysis and recorded on photographs. Measurements on the green and red fluorescent tracks resulted in a length of 17.7 ± 1.8 μm for BAC1 and

15.5 ± 1.2 μm for BAC3, with 3.7 ± 0.9 μm for the overlapping regions (Table 1). Taking into account the stretching degree (3.27 kb/μm) of the extended DNA fibres found previously (Fransz et al. 1996), we calculated the molecular sizes of BAC1, BAC3 inserts and the overlap to be 57.9 kb, 50.7 kb and 12.1 kb, respectively, which are similar to the physical sizes of the respective BACs as determined by PFGE (data not shown). Accordingly, the two partially overlapping BAC clones were estimated to cover a 95-kb ($57 + 50 - 12 = 95$ kb) contiguous genomic region around the *Mi* gene (see schematic physical map in Fig. 1c).

FISH mapping of *Mi*-BAC1 and *Mi*-BAC3 on pachytene chromosomes

To precisely localise the two BAC clones on the cytogenetic map of chromosome 6, we performed a two-coloured FISH experiment on pachytene chromosome preparations using digoxigenin-dUTP-labelled BAC1 and biotin-dUTP-labelled BAC3, respectively. After washing at high stringency ($0.1 \times$ SSC at 60°C), the BACs were found to be co-localised on the short arm of a chromosome that, on the basis of its morphology, was identified as chromosome 6 (Ramanna and Prakken 1967). Hybridisation signals appeared at the border between the euchromatic region and the pericentric heterochromatin (Fig. 1d) at a fractional length of the short arm of 0.4 relative to telomere. At late pachytene stage, this chromosome 6 has an average length of 36.9 μm (Fig. 1d) with 2.3 μm (short arm) and 23.6 μm (long arm) corresponding to the euchromatin, and 4.3 μm (short arm) and 6.7 μm (long arm) to the heterochromatin.

FISH mapping of *Aps-1* YAC

Hybridisation of biotinylated *Aps-1* YAC DNA to pachytene chromosomes of 'VFNT cherry' resulted in a prominent red signal in the pericentromeric heterochromatin of the long arm of chromosome 6, near the transition to euchromatin (Fig. 1e). In conjunction, these mapping studies show that the pericentromeric heterochromatin of tomato chromosome 6 is contained between the *Mi* locus on the short arm and the *Aps-1* locus on the long arm.

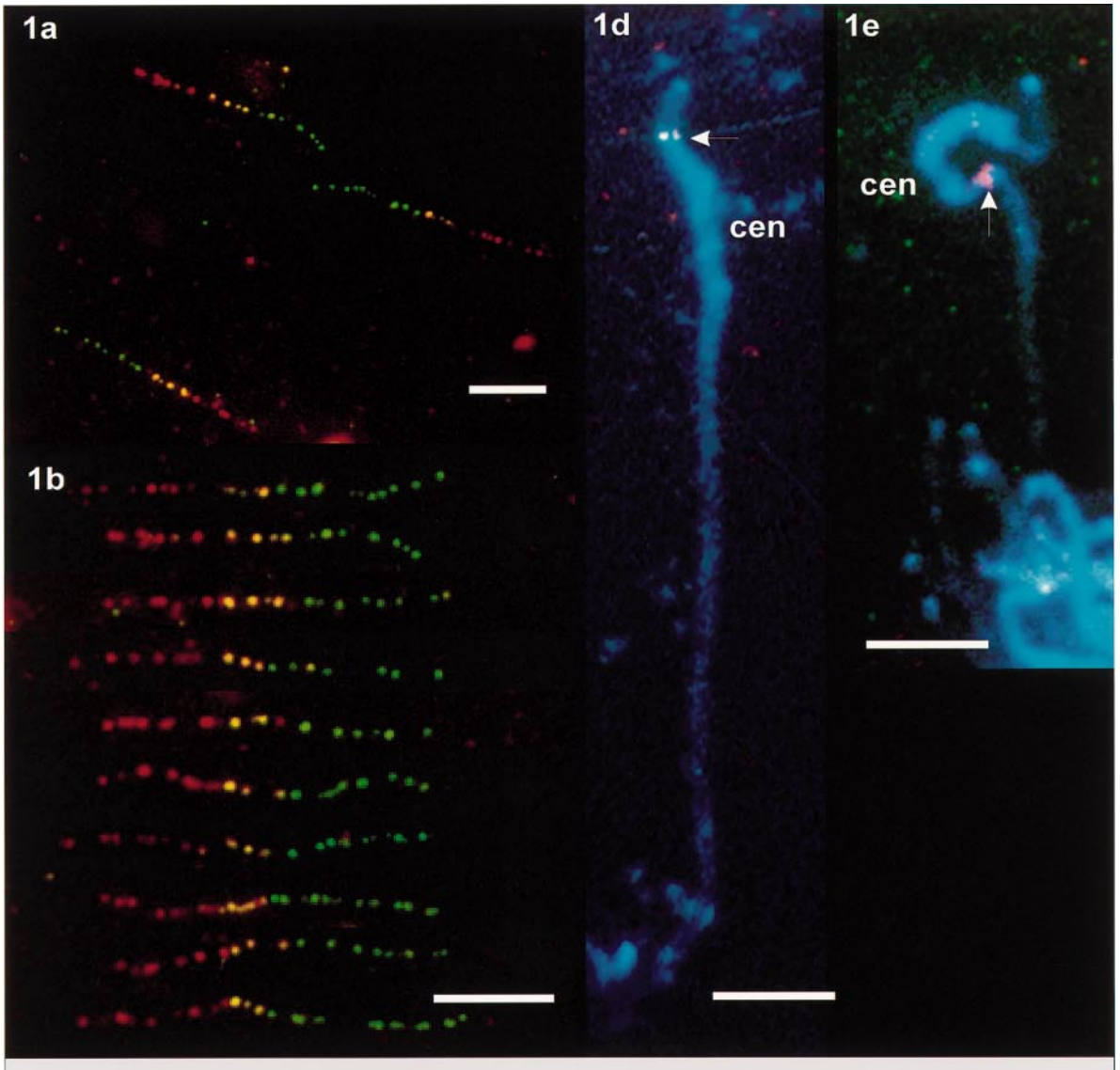
Discussion

In this paper we have provided direct physical evidence showing that the root-knot nematode resistance gene *Mi* is located on the short arm of chromosome 6, as already indicated by molecular mapping (see Fig. 2a, Liharska et al. 1997; also see van Wordragen et al. 1994, 1996), and not on the long arm as has long been anticipated on the basis of its very tight genetic linkage to the loci *Aps-1* and *yv* (see classical genetic map in Fig. 2b, Weide et al. 1993).

With a genetic distance of only 1.1 cM between *Mi* and *Aps-1*/*yv*, the actual position of *Mi* – at least 40 Mb away from these loci (see below) on the other side of the centromere near the telomere proximal border of the pericentromeric heterochromatin and euchromatin – is remarkable, even taking into account the pericentromeric location. Centromeric regions and their surrounding heterochromatin are notorious for their suppressive effect on meiotic recombination (Roberts 1965; Lambie and Roeder 1986) and are often associated with the clustering of genetically unresolvable markers on the molecular genetic linkage map (Tanksley et al. 1992).

The uneven distribution of recombination events along the chromosomal arms of tomato has recently been demonstrated by ultrastructural analysis of late recombination nodules (RNs) in synaptonemal complexes (SCs) of pachytene cells (Sherman and Stack 1995). As RNs are associated with the sites of crossing-over, the appearance of RNs directly reflects the distribution of recombination events along the chromosomes. As shown in Fig. 2D, among the 400 SCs analysed, virtually no RNs were found in the heterochromatic region of the short arm and only a few RNs were detected in the heterochromatin of the long arm. Accordingly, a 1-cM genetic distance between loci in the RN-free heterochromatin should correspond to a large physical distance.

An indication as to how far *Mi* is actually away from *Aps-1*/*yv* can be gained from recent estimates of the DNA content of hetero- and euchromatin regions. The tomato haploid genome has a DNA content of approximately 950 Mb (Arumuganathan and Earle 1991) packaged into 12 chromosomes spanning a total length of 483 μm at late pachytene stage (Ramanna and Prakken, 1967); therefore, the compactness of the tomato chromosomes is on average 2 Mb/μm. As approximately 77% of the genomic DNA is located in heterochromatin and the remainder in euchromatin (Peterson et al. 1996), thereby accounting for 116 μm and 367 μm of the chromatin lengths, respectively (Ramanna and Prakken 1967), DNA compactness in heterochromatin corresponds to 6.3 Mb/μm and in euchromatin to 0.6 Mb/μm. If we take the microscopic length measurements for chromosome 6 into consideration, the heterochromatin in the short arm accounts for at least 27 Mb of DNA, with *Mi* being located at the junction with euchromatin (Fig. 2c). Accordingly, the genetic distance of 1 cM between *Mi* and *Aps-1*/*yv* corresponds to a physical distance of more than 40 Mb, a base pair-to-centiMorgan relationship more than 50-fold higher than the average value of 750 kb/cM of the tomato genome (Tanksley et al. 1992).


1a
1b
1d
cen
1e
cen

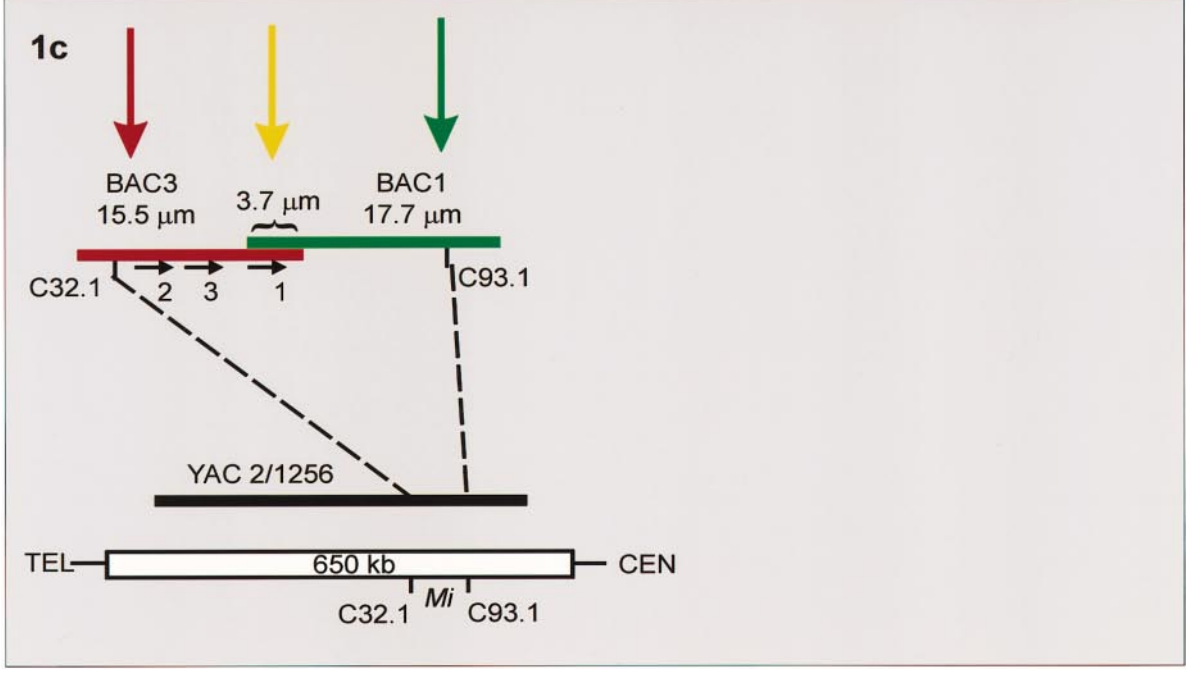

1c
BAC3
15.5 μm
3.7 μm
BAC1
17.7 μm
C32.1
2
3
1
C93.1
YAC 2/1256
TEL
650 kb
CEN
C32.1
Mi
C93.1

Table 1 Fibre-FISH signal lengths and molecular sizes of the two BAC DNA clones[a]

BAC	Number of observations	Length of the fluorescent signal (μm)	Molecular size based on fibre-FISH (kb)	Molecular size based on PFGE (kb)
BAC1	30	17.7 ± 1.8	57.9 ± 5.9	57
BAC3	30	15.5 ± 1.2	50.7 ± 3.9	50
Overlap	30	3.7 ± 0.9	12.1 ± 2.9	

[a] The signal lengths of BAC1, BAC3 and overlap were measured from their Fibre-FISH fluorescent hybridisation signals. The molecular sizes of the BACs and the overlap were derived from the lengths of the signals × the stretching degree of 3.27 kb/μm (Fransz et al. 1996)

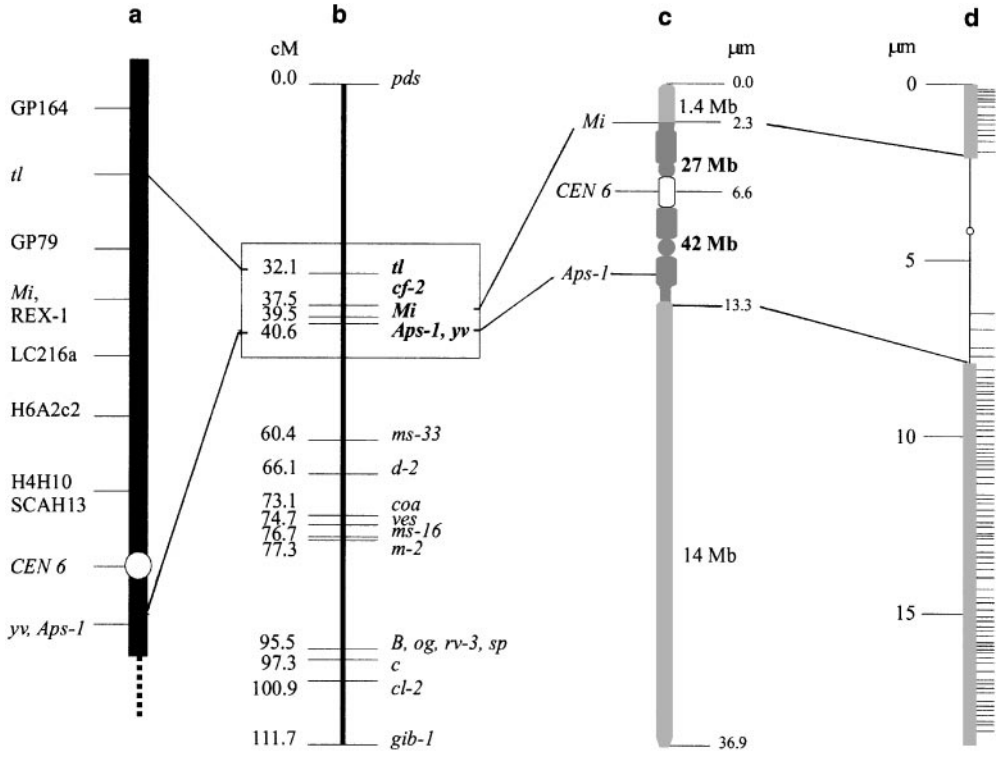


Fig. 2a Molecular map established by deletion mapping showing molecular marker order in the region around the centromere of tomato chromosome 6 (Liharska et al. 1997). **a, b** Classical genetic linkage map of tomato chromosome 6 showing genetic distances between classical markers (Weide et al. 1993). **c** Ideogram of the pachytene chromosome 6 showing the lengths and DNA contents in the euchromatin (*grey regions*) and heterochromatin (*black regions*) in both the short arm and long arm. The *Mi* gene is placed on the border of the euchromatin and heterochromatin of the short arm, while the *Aps-1* locus is in the pericentromeric heterochromatin of the long arm close to the junction with the euchromatin. **d** Recombination nodule map of tomato chromosome 6 showing the distribution of RN along the chromosome according to Sherman and Stack (1995). A *thick gray line* represents euchromatin and a *thin line* represents heterochromatin. The distance between each horizontal thin line equals one map unit measured from the centromere

Fig. 1a–c Physical mapping of BAC1, BAC3 and Aps-1 YAC by FISH. **a** Hybridisation signals of BAC1 (*green*) and BAC3 (*red*) to extended DNA fibres. Short stretches of *yellow* fluorescence indicates co-localisation of green and red signals and are interpreted as an overlapping region of the two BACs. **b** Ten hybridisation signals are digitally aligned based on position of overlaping parts. **c** A schematic representation shows the 650-kb introgressed *L. peruvianum* DNA containing the *Mi* region between the markers C32.1 and C93.1 with the physical map of the two BACs. The *arrows* indicate the position of three *Mi* homologs of which homolog 2 is the active one (Milligan et al. 1998). *Bar*: 10 μm. **d** Localisation of the BAC1 and BAC3 related to the *Mi* gene by FISH to pachytene chromosome 6. The *white spots* represent BAC1 and BAC3 hybridisation signals, co-localising at the border of euchromatic and heterochromatic regions in the short arm. The chromosome length is 36.9 μm. *Bar* 5 μm. **e** Localisation of the Aps YAC (*red signal*) near the transition of the pericentromeric heterochromatin and the euchromatin in the long arm of chromosome 6. *Bar*: 5 μm

Clearly, FISH mapping of specific tomato sequences to meiotic pachytene chromosomes adds an extra dimension to the molecular genetic linkage maps of tomato and should be regarded as being crucial before embarking on the positional cloning of a target gene merely on the basis of its very tight linkage to molecular markers. Moreover, the integrated cytogenetic-molecular map permits the estimation of distances up to megabase pairs between markers along the chromosome.

A fortunate circumstance emerging from the present FISH mapping data is that molecular access has been gained to a chromosomal region that otherwise would have remained difficult to identify molecularly, the transition of heterochromatin into euchromatin.

Conceivably, sequence analysis of the telomere and centromere proximal regions around *Mi* may provide important information on typical structural sequence elements defining euchromatin and heterochromatin in that chromosome.

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