TOMATO GENOME MAPPING BY FLUORESCENCE *IN SITU* HYBRIDISATION

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Kartering van het tomatengenoom met behulp van fluorescentie in situ hybridisatie

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
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Propositions

1. Chromosome mapping by fluorescence *in situ* hybridisation (FISH) is the most informative genome mapping approach, compared to genetic linkage and molecular mapping.
2. FISH to pachytene chromosomes is more effective in plants than in animals.
3. Tomato telomeres show a heterogeneous molecular organisation of unique repeat families at each individual chromosome end, implying that their unique distribution and repeat lengths are indispensable for important chromosome functions and behaviour.
4. Chromosome organisation in tomato and *Arabidopsis* differs mainly with respect to the size and positions of their heterochromatin regions.
5. Genes located near the border of heterochromatin and euchromatin are likely to be affected in expression and silencing because of their position.
6. In estimating the molecular size of a repeat domain Fibre-FISH generates more precise data than pulsed field gel electrophoresis.
7. The limitation of detection sensitivity of standard FISH has been broken through by using FISH in combination with rolling circle amplification that can detect target sequences as small as 20-30 bp and discriminate even a single point mutation.
8. Compared with the Dutch scientific society, the American scientists show stronger competitions, but fewer co-operations.
9. An old Chinese proverb says: "When you hear something, you will forget it. When you see something, you will remember it. But not until you do something, will you understand it." I believe this particularly applies to understanding a concept after doing biological experiments.
10. 不患人之不己知，患不知人也。　　孔子 - 论语

    Confucius said: "Do not be concerned about others not appreciating you. Be concerned about your not appreciating others." Although this verse came from the most famous Chinese philosopher about 2500 years ago, I think it is still true for a gentlemen today.

These propositions are a part of the thesis, "Tomato Genome Mapping By Fluorescence *in situ* Hybridisation" by Xiao-bo Zhong, Wageningen, Wednesday 13 May 1998.

Contents

Chapter 1

GENERAL INTRODUCTION: TOMATO GENOME MAPPING BY FLUORESCENCE *IN SITU* HYBRIDISATION (FISH)

Introduction

The genome is usually defined as the unique complement of hereditary material in the nucleus of an organism and represents the DNA content of a haploid set of chromosomes. In addition, each plant contains numerous minuscule extranuclear genomes found in mitochondria and chloroplasts. In this thesis, we will only refer to the nuclear genome. The nuclear genome is made up of extremely long DNA molecules, one for each chromosome, that have defined sequences along their length known as genes. Genome mapping involves localising DNA sequences or genes to their positions on the chromosomes.

The past decade has seen a major advance in genome mapping largely due to the development and application of molecular biological and genetical techniques, many of which are now in routine practice. Mapping is now being carried out on the whole range of genomes from the most simple, to ones that are extremely complex. Eukaryotic genome analysis (Miklos and Rubin, 1996) has mainly concentrated on several model organisms. Yeast has been the first eukaryotic genome to be sequenced completely (Oliver *et al.*, 1992; Dujon *et al.*, 1994; Johnston *et al.*, 1994; review by Dujon, 1996; also see the supplement to Nature 387 No. 6632: The yeast genome directory). "Human genome projects", underway in several countries, have attracted widespread public awareness (Guyer and Collins, 1995; Savill, 1997). In higher plants, prime model species for dicots and monocots are *Arabidopsis thaliana* and rice, respectively. Tomato (*Lycopersicon esculentum*) is another representative model species for genome mapping in higher plants thanks to the following features: (1) It is a diploid species with 12 pairs of chromosomes, naturally self-pollinating, and large quantities of seed that make it amenable to genetic analysis. Moreover, the plant structure allows detection of a vast array of hereditary alterations. (2) The 12 chromosomes are highly differentiated and identifiable at the meiotic prophase stages, rendering it a good species for cytological and cytogenetic studies. (3) It has a relatively small genome (950 Mb) that is open to manipulation by molecular biological and genetical tools. (4) Cells are readily cultured and whole plants can be regenerated and transformed by *Agrobacterium*-based vectors, thereby lending themselves to genetic engineering. (5) It is also an important economic crop worldwide that is used extensively for both basic and applied research.

Such advantages have made tomato one of the favourite model species for plant genome mapping over the past century by classical genetics, cytogenetics, molecular genetics and molecular biology. This chapter reviews the progress that has been made in tomato genome mapping and discusses the development of the new technique of fluorescence *in situ* hybridisation (FISH) in this context.

Classical genetic mapping of tomato genome

From Mendel's early experiments on pea in 1865 until the demonstration that DNA was the hereditary molecule (Avery *et al.*, 1944), genome analysis was carried out by means of classical transmission genetics. Mendel introduced the concept of phenotypic characters being determined by independent segregating elements of heredity. Tomato was one of the many organisms investigated by classical genetics. In 1905 Halsted *et al.* published their data on the genetic analysis of five distinctive morphological traits. However, Morgan's (1910) genetic linkage analysis in *Drosophila* was the real beginning of genome mapping by genetic analysis which showed the relative positions of genes, corresponding to the different phenotypic characters, on the map. The first linkage studies in tomato were made by Jones (1917) who demonstrated co-segregation of dwarfness (*d*) and elongate fruit shape (*o*) genes due to linkage. Linkage analysis is based on the concept, that certain genes co-segregate due to their being localised on the same chromosomes. Genes borne on homologous chromosomes belong to the same linkage group. Linkage of genes can be broken when homologous linkage structures exchange corresponding parts by crossing over during meiosis. The crossover leads to recombination of genes in the same linkage group. The frequencies of recombination are used as the basis for gene ordering. A 1% chance of genetic recombination between two genes is defined, in genetic distance, as 1 centimorgan (cM). Depending on the genome size of the organism, 1 cM represents on average a 139 kb sequence in *Arabidopsis*, 750 kb in tomato, and 1108 kb in human. The relative distances allow the unequivocal localisation of genes in particular linkage groups. In the 1950s the use of natural and radiation-induced tomato mutants led to a number of genes being systematically mapped on a linkage map (Butler, 1952). The morphological map of the tomato genome was further extended by Rick (1975) to 190 loci and additional loci are still being added.

Cytological and cytogenetic analysis of the tomato genome

Cytology, which is the study of the microscopic appearance of cells, nuclei and chromosomes, became feasible when it was demonstrated that the DNA specific dye pararosanilines-Schiff's reagent visualised chromosomes in fixed tissue (Feulgen and Rossenbeck, 1924). Prior to 1940, it was thought that as the somatic and meiotic metaphase chromosomes of tomato were very small and it was almost impossible to identify individual chromosomes under the light microscope, it would be a difficult species to use for cytological studies. However, Brown (1949) and Barton (1950) revealed that the tomato genome was packaged into 2n=2x=24 acrocentric to metacentric chromosomes and that the

greatly extended chromosomes at the meiotic pachytene stage displayed good morphological differentiation into euchromatic (poorly stained) and heterochromatic (densely stained) regions upon staining, with each arm terminating in a detectable telomere. Barton (1950) was the first to identify each of the 12 bivalents at the pachytene stage. Ramanna and Prakken (1967) further observed the structural differentiation and homologies between the pachytene and somatic metaphase chromosomes and identified the 12 pachytene bivalents and the 12 pairs of somatic metaphase chromosomes. Like chromosomes of other members of the *Solanaceae*, those of tomato have pericentric heterochromatin. On average, the pachytene chromosomes are 15 times less spiralised than metaphase chromosomes. However, the heterochromatin parts at this stage are extended only by a factor of 4 to 5 compared to metaphase stage, while the euchromatin parts do so by a factor of 30 (Ramanna and Prakken, 1967). By analysing Feulgen-stained pachytene chromosomes using densitometry and image analysis, Peterson *et al.*, (1996) investigated the amount of DNA in euchromatin and heterochromatin. The results suggested that 77% of the tomato genome is located in heterochromatin and 23% in euchromatin.

Cytogenetics focuses on the correlation of transmission genetics and chromosome number, morphology and behaviour during mitosis and meiosis. Cytogenetic mapping is a means to determine the locus of a specific gene with respect to the cytological landmarks of a chromosome. Such a locus may be detected on a specific chromosome arm or a small segment of such an arm. One of the most effective cytogenetic mapping techniques is deletion mapping, but requires the availability of cytological techniques to identify individual chromosomes, and an ability to generate sufficient deletion stocks, both requirements being met in tomato. The use of 74 radiation-induced deletions in tomato chromosomes, made it possible to assign the loci of 35 genes to 18 of the 24 arms, by identification of the deletion positions on the pachytene chromosomes (Khush and Rick, 1968). These findings established a cytogenetic map showing the centromere positions, orientation of linkage groups and markers on each chromosomal arm. Once molecular cloning techniques became available, deletion mapping could be used to assign DNA markers to specific chromosome regions, as shown for wheat (Werner *et al.*, 1992) and tomato (Liharska *et al.*, 1997).

Discrepancies between genetical maps and cytogenetical maps can be explained by non random distribution of crossover events along the chromosomes. Such a distribution can also be investigated microscopically by determining the frequency and distribution of recombination nodules (RNs) on the tomato synaptonemal complexes (SCs). Tomato demonstrates its superiority in exhibiting well defined RNs. A high-resolution physical recombination map for tomato chromosomes shows the non-random distribution of RNs along SCs (Sherman and Stack, 1995). There are no RNs at the distal 2% segments of SCs, in kinetochores, or in the nucleolus organiser at the short arm of chromosome 2. The RNs

are more common per unit length of SC in euchromatin compared to SC in heterochromatin. Such findings greatly improve our understanding of why genetic distances are not proportionally related to physical distances along chromosomes.

Although cytological and cytogenetical analysis expanded our knowledge of the tomato genome, it is only since the development of molecular biological techniques that a much clearer picture has emerged.

Molecular analysis of the tomato genome

Since it was demonstrated that DNA acted as the hereditary material (Avery *et al.*, 1944), molecular biological techniques have been developed quickly leading to a better understanding of its structure. Firstly, the size of the unreplicated haploid nuclear genome (known as the *C*-value) can be estimated by various means, including micro-densitometry of Feulgen-stained nuclei, DNA reassociation kinetics, nuclear volume measurements, and flow cytometry, or can be derived from sampling genomic clone libraries. These diverse methods provide only slightly different values for the same species. The *C*-value of the tomato genome was estimated to be 1.1 pg/1C or 950 Mb/1C by densitometry of Feulgen-stained nuclei (Anderson *et al.*, 1985). There is a large variation in size (up to 1500-fold) among plant species and tomato is a species with a rather small genome, only 7-8 times larger than the smallest plant genome known (*Arabidopsis thaliana*), twice that of rice, one-sixteenth of wheat, and 1/130 of lily which belongs to the species with largest plant genome size (Bennett and Smith, 1991).

Complex genomes of higher plants are characterised by numerous types of repetitive DNA sequences. With the use of DNA-reassociation kinetic studies (reviewed by Flavell, 1980), plant genomes were demonstrated to be composed of highly or moderately repeated (rapidly reannealing) and low- or single-copy (slowly reannealing) DNA sequences. Plants with a large genome are found to possess a much higher proportion of repeated DNA sequences than those with a smaller genome size (Flavell, 1980).

The first broad sketch of how the tomato genome is organised at the DNA sequence level was given by analysing 50 randomly selected genomic clones (Zamir and Tanksley, 1988). A large proportion of clones (78%) behaved as single copy under high stringency washing condition in Southern analysis, showing that the small tomato genome contains only a low proportion of repeated sequences. A number of different classes of repetitive DNA sequences in tomato have been identified by various methods. The first identified highly repetitive DNA sequence in tomato was a 452-bp *HindIII* repeat THG2 which is a member of a large, complex repeat dispersed throughout the genome (Zabel *et al.*, 1985). Several other major repeat classes in the tomato genome were further identified and

characterised with respect to copy number and chromosome location (Ganal *et al.*, 1988). A family of tandemly repeated sequences consists of the genes coding for the 45S ribosomal RNA. The 9.1 kb repeat unit in tomato was estimated to occur in approximately 2300 copies on a single locus at the end of the short arm of chromosome 2 in the nucleolar organiser region (NOR). The 5S ribosomal RNA gene cluster (450-600 kb in the tomato genome) has tandem repeat sequences with about 1000 copies of the unit and is located in the pericentromeric region of the short arm of chromosome 1 close to the centromere (Lapitan *et al.*, 1991; Xu and Earle, 1996a). Another family of highly tandemly repeated sequences with a unit length of 162 bp is TGR1, which has approximately 77,000 copies. This repeat is clustered at or near the telomeres of most chromosomes and also at interstitial sites of a few chromosomes. A dispersed repeat family, TGR2, was found on nearly all the chromosomes except chromosome 2 with an average of 133 kb between elements. The total copy number of this repeat is approximately 4200. Another repeat (TGR3) showed clustering at or near the centromeres in nine chromosomes and was present along the whole of the other three chromosomes, except for the distal regions. This repeat had a copy number of approximately 2100. TGR1 and TGR2 do not cross-hybridise to the closely related potato genome, demonstrating that these repeated sequences are evolving at a rate higher than most genomic sequences. TGR3 hybridises to all *Lycopersicon* species and some *Solanum* species (Ganal *et al.*, 1988).

The macrostructure of the tomato telomeres was also investigated by molecular methods (Ganal *et al.*, 1991). The chromosome ends carry a simple telomeric repeat (TR), with a 7-basepair unit of TT(T/A)AGGG which is almost identical to the TTTAGGG motiv of the *Arabidopsis* TR sequence. TGR1 was further characterised as a subtelomeric repeat that is localised at 20 out of 24 tomato telomeres. Along with spacers, TR and TGR1 represent the macrostructure of the tomato telomeres and account for approximately 2% of the total tomato genome.

The development of molecular cloning techniques, has enabled the establishment of specific molecular markers at defined places along each chromosome. These molecular markers can be used to determine the position of a particular gene of interest. The RFLP technique, referred to as restriction fragment length polymorphism (Botstein *et al.*, 1980), has resulted in an explosion of genetic mapping in plants. This technique uses cDNA or other cloned DNA elements as probes to detect differences between individuals, at the DNA level, resulting from nucleotide substitution or sequence rearrangements (insertion or excision). A high density RFLP map of the tomato genome was established with more than 1000 markers spanning a total of over 1276 centimorgan map lengths distributed along the 12 chromosomes (Tanksley *et al.*, 1992). In addition to the RFLP technique, a wide range of other techniques for detecting DNA markers has been developed, including randomly amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism

(AFLP). Such marker techniques have also been used to complement the RFLP map so as to provide an even higher density map in some specific regions of interest (van Wordragen *et al.*, 1996; Bonnema *et al.*, 1997). Marker-assisted breeding and map-based cloning of interesting genes, requires the availability of an accurate genetic linkage map showing the position of the trait of interest relative to molecular markers. In tomato, integration of molecular maps with classical genetic maps has been carried out for the top part of chromosome 1 (Balint-Kurti *et al.*, 1995), chromosome 3 (Van de Biezen *et al.*, 1994) and chromosome 6 (Weide *et al.*, 1993; Van Wordragen *et al.*, 1996).

The average relationship between genetic and physical distance in tomato is equal to 750 kb per cM (Tanksley *et al.*, 1992; Pillen *et al.*, 1996). However, the actual ratio of genetic and physical distance varies considerably depending on the chromosomal region under investigation. Particular regions have been estimated to show as little as 43 kb per cM near the *I*-2 locus which is located in the euchromatin, distant from the centromere of chromosome 11 (Segal *et al.*, 1992), to as much as over 5 Mb per cM around the *Tm*-2a region which is located close to the centromere of chromosome 9 (Ganal *et al.*, 1989). Different components of DNA do not participate equally in recombination, in particular the repetitive DNA elements, which account for much of the difference in genome size between different taxa.

Such distortion of the linkage genetic map in comparison to the physical size of the region leads to difficulty in isolating any particular gene. Therefore, an essential step to move from a linked marker towards a particular gene is to first create a physical map of the chromosomal target region. A relatively new approach which provides the means to correlate molecular maps to chromosomes is fluorescence *in situ* hybridisation (FISH). This technique is routinely applied to determine the locations of DNA elements on specific chromosome regions. Not only is this technique useful for correlating particular chromosomes to genetic linkage groups, but it is also the most appropriate tool for studying the distribution of repetitive DNA elements across the genome.

Genome mapping by fluorescence *in situ* hybridisation (FISH)

Development of the FISH technique

The *in situ* hybridisation (ISH) technique was initially developed to detect RNA or DNA distribution in cytological preparations by using isotopic labelled RNA or DNA probes (Gall and Pardue, 1969; John *et al.*, 1969; Buongiorno-Nardelli and Amaldi, 1969). With the development of molecular cloning techniques in the early 1980s, this technique was used to detect sequences, as small as 1 kb, in chromosomes at the metaphase stage

(Harper *et al.*, 1981; Rabin *et al.*, 1984). However, in spite of this sensitivity, the technique is disadvantageous by hazards associated with radioactive labelling, a long exposure time of several weeks or months, the need for extensive statistical analysis and the limited resolution by emitted isotopic signals captured by the emulsion layer. These drawbacks have been overcome by the substitution of radioactive procedures by non-isotopic *in situ* hybridisation (Rudkin and Stollar 1977; Langer *et al.*, 1981; Langer-Safer *et al.*, 1982; Manuelidis *et al.*, 1982; Landegent *et al.*, 1985). One of the most widely used procedures for labelling nucleic acids non-isotopically involves the incorporation of nucleotides modified with biotin (Langer *et al.*, 1981; Brigati *et al.*, 1983; Rayburn and Gill, 1985) or digoxigenin (Kessler *et al.*, 1990) into probes using nick translation or random primer methods. The labelled probes were initially detected *via* high-density coloured precipitates generated by enzymatic reporter molecules, commonly horseradish peroxidase or alkaline phosphatase conjugated avidin or anti-digoxigenin antibodies (Josephs *et al.*, 1984; Manuelides, 1985). Fluorescence *in situ* hybridisation (FISH) became the vogue following the discovery that a biotinylated probe could be detected *via* fluorescent reagents and visualised under a fluorescence microscope (Pinkel *et al.*, 1986). The FISH technique is nowadays preferred over enzymatic assays because of its better spatial resolution and the greater potential for simultaneous multi-probe analysis.

The number of probes which can be detected simultaneously is basically determined by the fluorochromes that can be distinguished spectrally. Using a blue fluorescent AMCA, a green fluorescent FITC and a red fluorescent TRITC, three DNA sequences can be simultaneously visualised by FISH in three different colours (Nederlof *et al.*, 1989). It is possible, however, to increase the number of detectable targets by the combination of different DNA labelling and detection systems with computing image processing technique. The availability of fluorescence microscopy with digital image analysis (Arndt-Jovin *et al.*, 1985), in combination with a digital imaging system for pseudo-colouring and merging images and probe labelling with two or more different fluorochromes has thus increased the number of target sequences to seven, using only three fluorochromes (Ried *et al.*, 1992; Wiegant *et al.*, 1993b). Each fluorochrome signal was firstly captured in a black-white image and the respective images were combined and pseudo-coloured using specific image analysis software. Probes can also be discriminated by a combination of colours in different ratios (Nederlof *et al.*, 1992). Different ratios of two fluorochromes could clearly distinguish between three different probes (Nederlof *et al.*, 1992). Labelling with different combinations and ratios of only three fluorochromes, enabled a whole set of all 24 different human chromosomes to be simultaneously detected in discrete colours by using a pool of human chromosome painting probes (Speicher *et al.*, 1996).

The great improvement in sensitivity and resolution of the FISH techniques in the last decade, has led to it being a powerful tool used to analyse the organisation of complex

genomes. A specific application of FISH is known as genome painting or genomic *in situ* hybridisation (GISH) with the use of total genomic DNA as probe in distinguishing parental chromosomes in sexual and somatic hybrids. Chromosomes from genetically very close species, such as those from barley and *Hordeum bulbosum* (Anamthawat-Jonsson *et al.*, 1993), and potato and tomato (Jacobsen *et al.*, 1995), can be identified in their interspecies or intergeneric hybrids by labelling total genomic DNA from one species and blocking with the DNA of other species. The GISH technique has proven its usefulness in traditional genome analysis to establish intergenomic homologous chromosome pairing (King *et al.*, 1994). Whereas in allopolyploid species, using genomic DNA from putatively ancestral species, GISH can provide important information about the genome evolution and divergence of allopolyploid species (Bennett *et al.*, 1993), it is also a very useful tool to detect intergenomic translocations (Kenton *et al.*, 1993; Jellen *et al.*, 1994). Once a chromosome specific library is available which may be obtained from a large-scale flow-sorted fraction of metaphase chromosomes or by micro-dissection of chromosomes (or chromosome segments) and subsequent amplification of their DNA by PCR, FISH using such a chromosome specific library as probe, offers a tool for comparative analysis of chromosome homologies among closely related karyotypes. FISH with human chromosome specific DNA libraries revealed the sequence homologies in human and *Macaca fuscata* chromosomes (Wienberg *et al.*, 1992) and provided evidence for genomic rearrangements among humans, great apes and gibbons (Jauch *et al.*, 1992).

Another major successful application of the FISH technique is the physical mapping of repetitive and single-copy DNA sequences in the karyotypes of many species. A large number of repetitive DNA sequences from animal and plant species have thus been localised on specific chromosome regions by FISH (see review of Joos *et al.*, 1994; Jiang and Gill, 1994). This information significantly improves our knowledge of the molecular structure of chromosomes. Some tandemly repeated DNA sequences are mainly localised in the heterochromatin knobs at the telomere regions (Vershinin *et al.*, 1995; Zhong *et al.*, 1996a), whereas other repetitive DNA sequences are dispersed along the chromosomes, although restricted to heterochromatin regions. A number of centromere-specific repeated sequences were identified since they were mapped to the centromere regions by FISH (Haaf and Ward, 1994b; Fransz *et al.*, in press).

Physical mapping by FISH

The aforementioned properties of FISH showed that this tool is particular suitable for the direct construction of a chromosome (physical) map. Initially, the FISH technique was applied to metaphase chromosomes to assign clones to specific chromosome regions and to order clones along chromosomes (Lawrence *et al.*, 1988). More precise physical mapping

was obtained by applying FISH to stretched free chromatin (Heng *et al.*, 1992), extended genomic DNA fibres from interphase nuclei of humans (Wiegant *et al.*, 1992; Parra and Windle, 1993) and plants (Fransz *et al.*, 1996a), and stretched individual DNA molecules from isolated clones (Weier *et al.*, 1995). Two major factors have to be considered when FISH is used to construct a physical map. Firstly, what size target sequence can be detected by the probes., i.e. what is the sensitivity of the technique? Secondly, what is the mapping resolution of the technique, i.e. what distance between two probes can be resolved along the DNA molecules on different targets? Use of FISH on different hybridisation targets with DNA showing a varying degree of condensation, has resulted in a wide range of mapping resolutions from several megabases to only a few hundred basepairs and different sensitivities for target sizes from several kilobases to less than a hundred basepairs.

Use of FISH with cosmid probes of average molecular size of 30 kb enabled construction of a chromosomal map of metaphase chromosomes (Lichter *et al.*, 1990). The relative positions of different probes along the chromosomal axis were determined by measuring the fractional length (FL) value which indicates the distance from the hybridisation signal to a fixed reference point compared to the total length of the chromosome. Using cosmid clones, specific hybridisation signals existing on both chromatids of the two homologues in more than 80% of the metaphase complements have been reported (Lichter *et al.*, 1990). Smaller probe sizes ranging from a few kb to less than 1 kb, could be detected on human metaphase chromosomes although only 20-50% of the metaphase were informative (Richard *et al.*, 1994; Heppell-Parton *et al.*, 1994). However, detection of a small, single copy sequence on metaphase chromosomes is more difficult in plants than in human, because of the relatively highly condensed chromosome structure and the presence of a cell wall that might hamper target DNA accessibility. Although small single-copy probes of a few kb sequences were detected on petunia metaphase chromosomes (Fransz *et al.*, 1996b; Hoopen *et al.*, 1996), FISH on plant metaphase chromosomes is still mainly applied to detect highly repetitive sequences or large single copy targets using probes like yeast artificial chromosomes (YAC), or bacterial artificial chromosomes (BAC) (Jiang *et al.*, 1995). Use of FISH can only give a limited mapping resolution with metaphase chromosomes. Only when two target sequences are separated by a distance of more than 1-2 Mb can the hybridisation signals from two probes be spatially resolved (Lichter *et al.*, 1990).

To improve the spatial resolution of gene mapping and accessibility of chromosomal DNA targets to probes, a more decondensed chromosome preparation should be considered to replace the highly condensed metaphase chromosomes in plants, especially for species with a small genome sizes such as *Arabidopsis*, rice and tomato. Pachytene chromosomes, which are much less condensed than metaphase chromosomes, are better material for the development of a physical map by FISH (Shen et al., 1987; Albini and Schwarzacher,

1992; Fransz *et al.*, in press). Tomato chromosomes at the pachytene stage are, on average, 15 times longer than at the metaphase stage and should give a higher resolution physical map and have a higher sensitivity for smaller probes.

To further improve the mapping resolution for closely linked clones, FISH has been used on preparations of free chromatin (Heng *et al.*, 1992) and extended genomic DNA fibres from interphase nuclei (Wiegant *et al.*, 1992; Parra and Windle 1993; Fransz *et al.*, 1996). Free chromatin is released from interphase nuclei by using different chemical agents and a simple alkaline lysis procedure. Using such samples, FISH of several, closely linked single copy probes spanning a DNA size of up to several millions of base pairs demonstrates that the resolution of this technique is about 10 kb (Heng *et al.*, 1992). Yet, the free chromatin still retains its DNA-protein complex structure. A stronger lysis procedure releases free DNA from the interphase nuclei to form a DNA halo-loop or long extended-fibrilar structure. The technique involves the release of DNA fibres from lysed nuclei, followed by stretching them across the surface of a microscope slide to have a stretching degree between 3.0-3.5 kb/µm. Such highly extended DNA targets allow fine mapping of YACs, BACs, cosmids, lambda clones and plasmids, covering DNA stretches ranging from a few kilobases to a million base pairs at an accuracy of less than few kilobases and a detection sensitivity of a few hundred basepairs (Wiegant *et al.*, 1992; Parra and Windle, 1993; Haaf and Ward, 1994a and 1994b; Fransz *et al.*, 1996a).

Fibre-FISH was initially developed for human cells (Wiegant *et al.*, 1992; Parra and Windle, 1993) and further successfully adapted to plant species (Fransz *et al.*, 1996a). In the last 5 years it has become a powerful technique (Haaf and Ward 1994a and 1994b; Florjin et al., 1995; Nikali *et al.*, 1997). Fibre-FISH has now also been applied to individual DNA molecules rather than DNA fibres from nuclei (Weier *et al.*, 1995). The target DNA molecules in a solution of YACs, BACs, cosmids and lambda clones can be linearised and uniformly stretched on a flat glass surface coated with a monolayer of silane molecules by a procedure called "molecular combing" (Bensimon *et al.*, 1994). The DNA molecules prepared in this manner are remarkably linearised and homogeneously stretched at 2.3 kb/µm. By applying FISH directly to the stretched DNA molecules, fast mapping of λ DNA restriction fragments was feasible along the linearized 48.5 kb long phage λ DNA molecules with ~1 kb precision (Weier *et al.*, 1995). This technique also enables precise localisation and ordering of clones, resolves overlaps and distances, and provides a detailed picture of the integrity and colinearity of probes on individual YAC molecules as hybridisation targets (Weier *et al.*, 1995; Rosenberg *et al.*, 1995). Similarly, a recently developed procedure called "dynamic molecular combing" (Michalet *et al.*, 1997) permits the whole yeast or human genomic DNA to be stretched rapidly into irreversibly fixed, parallel running DNA fibres. This approach yields a high density of fibres with most DNA fragments longer than several 100 kb. By using FISH to such combed DNA molecules

direct mapping of probes to their respective position along the fibres is possible. With advantages, such as a high density of fibres, fast scanning and recording of signals, rapid measurements of numerous signals, this technique allows reliable distance measurements by statistical analysis without reference to any other method or internal control.

Before the application of the FISH technique to physical mapping, there was a gap between established genetic maps, cytogenetic maps and sequence-ready clones. Genetic maps only show the relative position of morphological and molecular markers along chromosomes on the basis of recombination frequency. Cytogenetic maps show chromosomal banding patterns and the relative positions of specific genes. Conventional means of physical mapping, such as contig ordering of large insert clones of YACs and BACs, and restriction mapping of redundant sets of smaller clones, can only give information of sequence distances in basepairs for isolated clones in a certain area. By bridging the gap between genetic maps, cytogenetic maps and physical maps, FISH techniques, especially Fibre-FISH, have either hastened these physical mapping steps or resolved the position of sequence-ready clones on specific chromosomal regions. By applying simple FISH of about one thousand YAC clones to metaphase chromosome preparations, the cytogenetic, genetic, and physical maps of about one-third of the human genome were integrated to show the relationship of genetic linkage distances to their physical locations (Bray-Ward *et al.*, 1996).

In the preceding sections, physical mapping by FISH of various DNA targets having different degrees of condensation has been discussed. It seems clear that a combination of FISH techniques, using targets of different density, from highly condensed metaphase chromosomes to completely free DNA molecules, provides an important supplementary tool to the more usual molecular methods employed for physical fine mapping.

FISH mapping in tomato genome analysis

Despite FISH having been widely applied, especially in human molecular cytogenetics, so far there have been no general applications of FISH to tomato genome analysis, even though tomato is a good model species for classical and molecular genetics. The first *in situ* hybridisation work in tomato involved the localisation of a biotin-labelled, dispersed repeat sequence THG2 to pachytene chromosomes (Zabel *et al.*, 1985). Four classes of repetitive sequences from the tomato genome were further mapped using a metaphase chromosome preparation by *in situ* hybridisation (Ganal *et al.*, 1988). The somatic chromosome karyotype of tomato was characterised on the basis of *in situ* hybridisation of the TGR1 satellite repeat (Lapitan *et al.*, 1989). Organisation of the telomeres and the 5S ribosomal RNA genes was studied by *in situ* hybridisation (Ganal *et al.*, 1991; Lapitan *et al.*, 1991). All this work was carried out by labelling the probes with

biotin and detecting the hybridisation signals with coloured immuno-chemical precipitates. Genomic *in situ* hybridisation techniques have been used to investigate tomato chromosome behaviour in somatic hybrids between tomato and potato (Wolters *et al.*, 1994; Jacobsen *et al.*, 1995). The FISH technique has also been applied to localise 45S, 5S ribosomal genes and YAC sequences to metaphase and pachytene chromosomes (Xu and Earle, 1994, 1996a and 1996b; Fuchs *et al.*, 1996) and to analyse the molecular organisation of 45S and 5S ribosomal genes on extended DNA fibres (Fransz *et al.*, 1996a). In this thesis, we describe the FISH techniques on both pachytene chromosomes and extended DNA fibres and discuss their applications to the analysis of the tomato genome structure and physical mapping of interesting genes to specific chromosome regions.

Scope of this thesis

This general introduction has reviewed the progress in tomato genome mapping using classical genetics, cytogenetics, and molecular genetics, emphasizing the great potential of FISH techniques.

Chapter 2 describes how to make preparations of mitotic metaphase chromosomes and pachytene chromosomes for FISH. To demonstrate the techniques, three classes of repetitive DNA sequences of 45S rDNA, TGR1 and THG2 are studied in single and multicolour FISH.

Chapter 3 describes two detailed FISH protocols for high-resolution physical mapping of DNA sequences in the tomato genome. The first technique involves FISH of pachytene chromosomes prepared from pollen mother cells and proves to be an excellent method for assigning DNA sequences to specific chromosome regions at a resolution of a few 100 kb. The second protocol presents a FISH technique to extended DNA fibres prepared from interphase nuclei with an even higher resolution of a few kilobases. This technique permits direct ordering of different clones and the study of molecular organisation of repetitive DNA sequences.

By using FISH with pachytene chromosomes and extended DNA fibres in combination with pulsed field gel electrophoresis, the molecular and chromosomal organisation of individual telomere domains are described in chapter 4. The two major repetitive sequences of the telomeric repeat (TR) and the subtelomeric repeat TGR1 are analysed by FISH in detail .

Chapter 5 describes a novel strategy for physical mapping of the TR/TGR1 organisation on chromosome 6 by employing a monosomic addition line in a tetraploid potato background selected from BC2 plants of a somatic hybrid between tomato and potato. FISH to extended DNA fibres and PFGE elucidated the molecular sizes of the

TR/TGR1 domain on the short arm and the interstitial TGR1 site on the long arm of that chromosome. Additionally, a dynamic feature of the TG and TGR1 sequences was discovered in the monosomic addition and its parental BC1 and the somatic hybrid, showing the recombinant nature of the alien chromosomes.

Chapter 6 describes the physical mapping by FISH to pachytene chromosomes and extended DNA fibres of two bacterial artificial chromosome clones spanning the nematode resistance gene *Mi*, to the border of the heterochromatin and euchromatin on the short arm of tomato chromosome 6.

In chapter 7, the current achievements of tomato genome mapping by FISH are discussed with respect to: (i) the mapping resolution and detection sensitivity of FISH techniques; (ii) dissection of the tomato genome by FISH; and (iii) a future perspective to construct a full bar-code map of each tomato chromosome.

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Chapter 2

PREPARATION OF TOMATO MEIOTIC PACHYTENE AND MITOTIC METAPHASE CHROMOSOMES SUITABLE FOR FLUORESCENCE *IN SITU* HYBRIDISATION (FISH)

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Abstract

Fluorescence *in situ* hybridisation (FISH) is an increasingly powerful tool with a variety of applications in both basic and applied research. With excellent genetic, cytogenetic and molecular maps available, the tomato genome provides a good model to benefit from the full potential of FISH. Tomato chromosomes at mitotic metaphase are small and not particularly suitable for high resolution FISH. In contrast, chromosomes at meiotic pachytene are about 15 times longer, and easier to identify by their differences in chromosome arm lengths and chromomere pattern. We have developed a technique for preparing chromosomal spreads of young pollen mother cells at mid-prophase I which is suitable for FISH. In a first series of experiments, the hybridisation patterns of three classes of repetitive DNA sequences were studied in single and multicolour FISH.

Keywords: tomato, pachytene, metaphase, fluorescence *in situ* hybridisation, FISH, repetitive sequences

Introduction

With the advent of the fluorescence *in situ* hybridisation (FISH) technique, cytogenetics has become instrumental in analysing the molecular organisation of eukaryote chromosomes (for review see Heslop-Harrison 1991; Joos *et al.*, 1994). In animal and human systems, multicolour FISH has thus been applied to construct cytogenetic maps showing the positions and order of molecular probes along the chromosomes, including cosmids (Lichter *et al.*, 1990; Inazawa *et al.*, 1994), YACs (yeast artificial chromosomes) (Marrone *et al.*, 1994; Selleri *et al.*, 1992; Moir *et al.*, 1994) and small, single-copy probes (Heppell-Parton *et al.*, 1994; Muleris *et al.*, 1994). As for plants, FISH has seen so far different types of applications (for review see Jiang and Gill 1994), ranging from the detection of alien chromosomes or segments in cereals (Schwarzacher *et al.*, 1992a; Mukai *et al.*, 1993) and the detection of parental chromosomes in (a) symmetric somatic hybrids (Parokonny *et al.*, 1992; Schwarzacher *et al.*, 1992b; Wolters *et al.*, 1994; Jacobsen *et al.*, 1994), to the mapping of repetitive and low copy DNA sequences in various species (Leitch *et al.*, 1991; Maluszynska and Heslop-Harrison 1991; Albini and Schwarzacher 1992; Xu and Earle 1994).

Tomato, with its excellent cytogenetic, genetic and molecular maps (Khush and Rick 1968; Tanksley *et al.*, 1992), provides a good model for applications of FISH. In focusing our attention to chromosome 6, we have recently succeeded in integrating the molecular

and classical linkage data into a combined map (Weide *et al.*, 1993; Van Wordragen *et al.*, 1994). With the final goal of developing a cytogenetically based physical map of chromosome 6 that further combines the order of loci from the molecular/genetic linkage map with cytological markers, we have started a molecular cytogenetic analysis of the tomato genome using FISH. As tomato chromosomes at mitotic metaphase are too condensed (2-4.5 μm) for high resolution physical mapping, such a map should be constructed using chromosomes at pachytene stage exploring their distinct morphology and highly differentiated pattern of euchromatin and heterochromatin (Ramanna and Prakken 1967). In this report, we present a technique for preparing chromosome spreads of young pollen mother cells at mid-prophase I which is particularly suitable for applications in FISH. As an example, the mapping of three classes of repetitive DNA sequences is described.

Materials and methods

Lycopersicon esculentum cv Cherry was used in all experiments. The following DNA clones were selected for probe labelling: (1) rDNA: an 0.7 kb petunia genomic DNA fragment derived from the 5' end of the 25S rRNA gene (Van Blokland *et al.*, 1994); (2) TGR1: a 162 bp telomere-associated satellite repeat (Ganal *et al.*, 1988), localised on most of the distal chromosome ends (Lapitan *et al.*, 1989); (3) THG2: a 452 bp repeat member of a large, complex dispersed repeat (Zabel *et al.*, 1985).

Young anthers about 3-4 mm long were selected for meiotic chromosome preparations. The stage of development was determined routinely in an acetocarmine squash preparation using a single anther from a flower bud. If at prophase I, the remaining anthers were fixed directly in acetic acid - ethanol (1:3) for 15-30 minutes, rinsed in deionised water and then incubated at 37°C for 2-3 hours in a mixture of pectolytic enzymes containing 0.3 % cytohelicase (Sepracor, France), 0.3 % cellulase "Onozuka" RS (Yakult Honsha Co., Ltd, Tokyo, Japan) and 0.3 % pectolyase Y-23 (Sigma P3026) in 10 mM citrate buffer, pH 4.5. After two washes in deionised water, the anthers were carefully transferred onto grease-free slides, and the pollen mother cells were dissected out of the anthers into a 1 μl droplet of water using fine-mounted needles, thereby taking care to remove supporting tissue as much as possible. Then 5 μl of 60 % acetic acid was added and the pollen mother cells were left for 1-2 minutes until the cytoplasm became sufficiently clear. A rim of freshly prepared, ice-cold (0°C) fixative was put onto the slide around the droplet containing the meiotic cells. Shortly after the fixative got mixed with the cell suspension, the cells were spread on the slide by adding some more drops of fixative on top of the material. Immediately after this treatment, the slide was briefly immersed in absolute ethanol, and then left to dry. The slides could be used directly for the *in situ* hybridisation or were stored at -20°C for up to several months.

Slides with mitotic metaphase complements were obtained from root tip meristems. The root tips from 2-4 week old plants were pre-treated in the spindle inhibitor 8-hydroxyquinoline (2 mM) for 2.5 hours at 17°C and fixed in acetic acid-ethanol (1:3) fixative for at least 15 minutes. Further treatments were as

described for the meiotic cells, though the enzymatic incubation was limited to 1 - 2 hours in a mixture containing only 0.1% of the three enzymes.

Probe DNA (1 µg) was labelled with either biotin-16-dUTP or digoxigenin-11-dUTP by random primer or nick translation labelling using the protocols of the manufacturer (Boehringer Mannheim, FRG). The *in situ* hybridisation protocol was carried out according to Wiegant *et al.*, (1991) with some modifications. The slides were pre-treated with 100 µg/ml DNase-free RNase A in 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0) at 37°C for 1 hour and then washed 3 times for 5 minutes in 2 × SSC. Then, the slides were incubated in 5 µg/ml (20 units/ml) pepsin in 0.01 M HCl for 7-10 minutes at 37°C, washed 3 times in 2 × SSC for 5 minutes, treated in 1% (w/v) alkaline formaldehyde (in borate buffer, pH 8.6) for 10 minutes at room temperature, washed 3 times in 2 × SSC for 5 minutes, dehydrated in a graded ethanol series (70, 90 and 100%), and finally air dried. The hybridisation mixture (20 µl per slide, containing 50% formamide, 2xSSC, 10% sodium dextran sulphate, 50 mM phosphate buffer pH 7.0, 1-2 ng/µl probe DNA and 50-100 ng/µl salmon sperm DNA) was added onto the pretreated chromosome preparations and heated to 80°C for 2 minutes to denature the probe DNA and the chromosomal DNA. *In situ* hybridisation was allowed to proceed at 37°C overnight, followed by post hybridisation washes for 3 × 10 minutes in 50% formamide, 2 × SSC pH 7.0 at 42°C, 10 minutes in 2 × SSC at room temperature, 3 × 10 minutes in 0.1 × SSC at 56°C and 10 minutes in 2 × SSC at room temperature.

Detection and amplification was according to the manufacturers protocols (Boehringer Mannheim). Digoxigenin-labelled probes were detected with anti-digoxigenin-fluorescein and amplified with rabbit-anti-sheep-fluorescein (F135, Nordic). Biotin-labelled probes were detected with Avidin Texas Red and amplified with biotin-conjugated goat-anti-avidin and avidin Texas Red. Chromosomes were counterstained with DAPI (5 µg/ml in McIlvaine buffer, pH 7) or propidium iodide (5 µg/ml in water). The slides were mounted in Vectashield (Vecta Laboratories) antifade mounting. The hybridisation signals were studied in a Zeiss Axioplan microscope equipped with Plan Neofluar optics and epi-fluorescence illumination using the filter sets 01, 09 and 14 for DAPI, FITC and TRITC, respectively. Photographs were taken on 400 ISO colour negative film. The images obtained from simultaneous hybridisation with two different probes were captured with a high sensitive CCD camera (Extended Isis, Photonic Science Ltd., UK) or scanned from negatives, merged and pseudocoloured using appropriate image processing software.

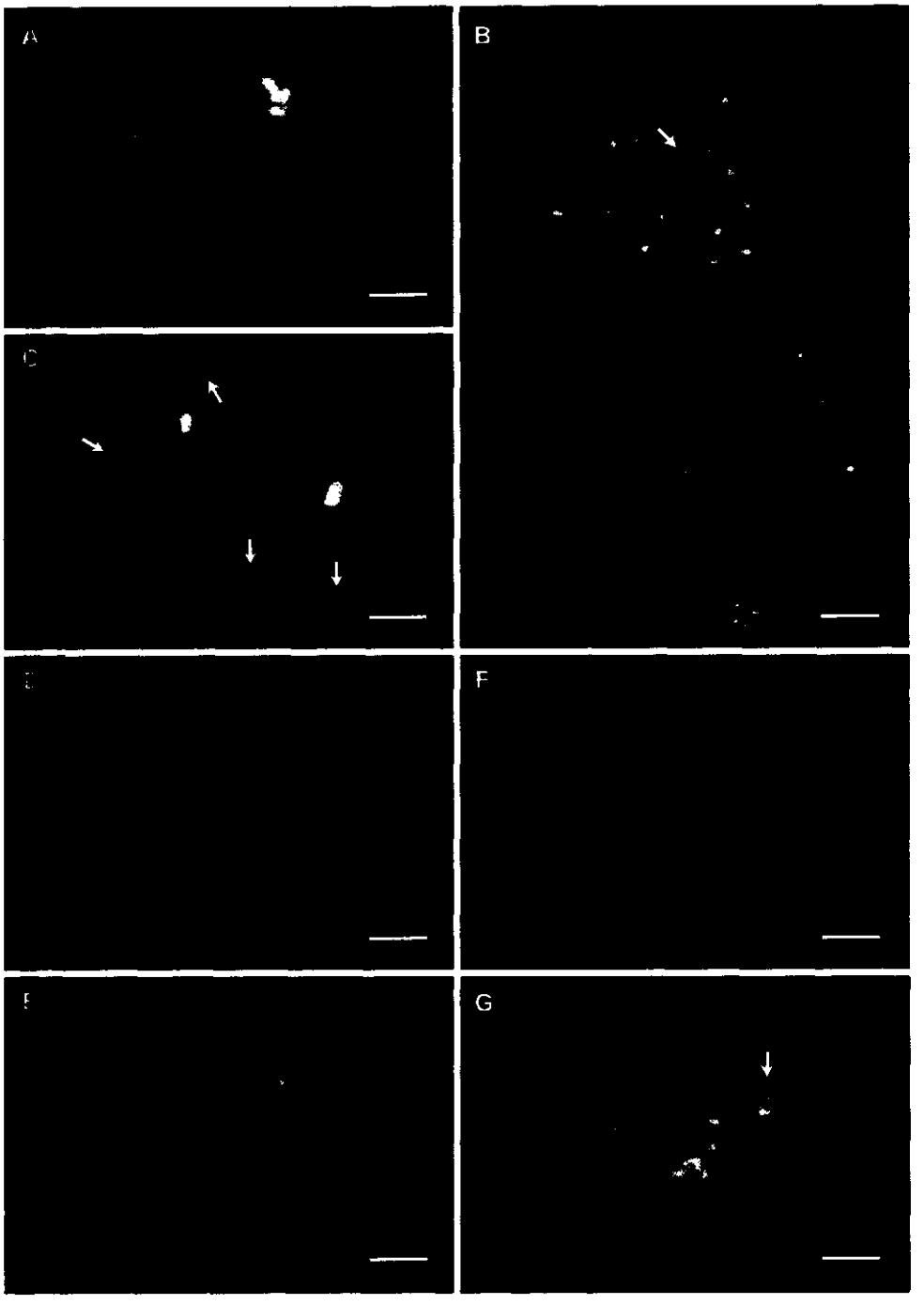
Results

Successful localisation of DNA sequences on chromosome spreads requires preparations of high quality. This is even more true for meiotic cells where remnants of thick callose walls and cytoplasm generate higher levels of background and reduced hybridisation signals. Therefore, our initial experiments were primarily focused on improving the technique for combining optimal spreading of well differentiated chromosomes with limited cell damage and clear background. Crucial improvements were found in:

(i) limiting the fixation time of the anthers to only 15 to 30 minutes;
(ii) prolonging the digestion with highly purified pectolytic enzymes to achieve complete breakdown of the callose walls without any risk of affecting the chromatin integrity;
(iii) controlled spreading of the cells using ice-cold acetic acid-ethanol fixative. Unlike the traditional squashing method, cell spreading protocols in tomato generally caused less chromosome loss and damage and resulted in an increase in the number of properly spread and complete meiotic prophase nuclei that were virtually free of cytoplasm;
(iv) briefly washing the slides in absolute ethanol immediately after cell spreading to further clear up the background.

Tomato chromosomes at pachytene are approximately 15 times longer than at mitotic metaphase (Ramanna and Prakken, 1967) and exhibit a well differentiated pattern of eu- and heterochromatin, which, along with chromosome length and centromere position, permits the identification of all 12 pairs. Upon DAPI or propidium iodide counterstaining, this pattern is largely retained, showing prominent fluorescing blocks at the pericentromeric regions and the telomeres, along with faint fluorescence at the euchromatic parts and the centromeres (see Fig. 1A, 1B and 1E). The satellite of chromosome 2, which is quite large in the tomato cultivar used, showed a brighter fluorescence, likely reflecting the presence of AT-rich highly repetitive DNA sequences (see arrows in Fig. 1B and 1E).

Figure 1. Fluorescence *in situ* hybridisation patterns of three repetitive sequences on metaphase and pachytene chromosomes of tomato, *Lycopersicon esculentum* cv VFNT Cherry. **A.** FISH of rDNA on pachytene chromosomes. The rDNA probe was labelled with digoxigenin and detected with anti-digoxigenin-FITC. **B.** FISH of TGR1 on pachytene chromosomes. The TGR1 probe was labelled with digoxigenin and detected with anti-digoxigenin-FITC. The arrow indicates the large heterochromatin satellite region at the short arm of chromosome 2. **C.** Simultaneous FISH of rDNA and TGR1 on metaphase chromosomes. The rDNA probe was labelled with digoxigenin and detected with anti-digoxigenin-FITC (green) and the TGR1 probe was labelled with biotin and detected with Avidin Texas Red (red). The three colour image has been obtained by merging and pseudocolouring the FITC, Texas Red and DAPI images using appropriate image processing software. The arrows indicate the chromosomes without the TGR1 signals at the ends of the long arms. The chromosomes in **A. B. C.** were counterstained with DAPI. **D. E.** and **F. G.** FISH of THG2 on metaphase and pachytene chromosomes, respectively. The THG2 probe was labelled with biotin and detected with avidin Texas Red. The chromosomes were counterstained with DAPI. The arrow in **G** indicates the large heterochromatin satellite region at short arm of chromosome 2. Bar equal to 5 μm in **A. C. D. E. F.** and **G.** and 2.5 μm in **B.**


A
B
C
D
E
F
G

To establish optimal conditions for FISH, pachytene chromosome preparations were subjected to hybridisation with some control probes of known chromosome location. Hybridisation with digoxigenin labelled rDNA, followed by detection with anti-digoxigenin-FITC, revealed a strong and specific green fluorescent signal at the large distal block of the satellite chromosome of chromosome 2 (Fig. 1A). The second probe tested was the telomere-associated satellite sequence TGR1, a 162 bp repeat shown to be located on 40 out of the 48 chromosome ends and on some interstitial sites of mitotic metaphase chromosomes (Ganal *et al.* 1988, Lapitan *et al.*. 1989). Simultaneous hybridisation of biotin labelled TGR1 and digoxigenin labelled rDNA probes on metaphase chromosomes showed bright red fluorescent signals of TGR1 at 40 of the 48 chromosomal ends (Fig. 1C). No TGR1 signals were detected on chromosome 2 (identified by carrying the green signals of the rDNA probe), whereas two pairs of chromosomes only exhibited signals at their respective short arms (see arrows in Fig. 1C). FISH of digoxigenin labelled TGR1 on pachytene chromosomes (Fig. 1B) revealed 20 out of 24 pachytene bivalent ends with green TGR1 signals. There was a striking difference in size of the TGR1 signals, suggesting a considerable variation in sequence length of this satellite repeat among the chromosomes. The pachytene bivalents not only showed a distribution of distal TGR1 signals comparable to that of the metaphase chromosomes (Fig. 1C), but they also revealed small TGR1 spots at some interstitial sites.

The 452 bp Hind III repeat THG2 is a cloned member of a large, complex dispersed repeat family specific for the tomato genome (Zabel *et al.*, 1985, Wolters *et al.*, 1991). Its distribution pattern was studied in preparations containing spread nuclei from both pollen mother cells at pachytene (Fig. 1F and 1G) and their accompanying endopolyploid tapetal cells (Fig. 1D and 1E) using biotin labelled THG2 probe. Hybridisation on the highly condensed metaphase chromosomes indicated that this repetitive sequence occurs on all chromosomes, but that the number of copies differs considerably among the individual chromosomes. Here again, hybridisation on pachytene chromosomes showed superior resolution. Signals were mainly confined to the proximal heterochromatin regions (Fig. 1F).

Discussion

Our focus was primarily on screening current chromosome techniques for their ability to yield high numbers of meiotic nuclei from dissected anthers and to spread their chromosomes without notable loss and damage. We have improved the cell spreading and subsequent treatments in such a way that the characteristic differentiation of euchromatin and heterochromatin segments of pachytene bivalents, as described for carmine or Feulgen stained squash preparations, was fully retained in the DAPI or propidium iodide stained chromosomes. Such differentiation is indispensable for the identification of all twelve

bivalents and, thus, for assigning molecular markers and repetitive sequences by FISH to specific regions of the cytogenetic map.

The physical resolution of chromosomes at pachytene stage is, on average, 15 times higher than at metaphase stage, but differs significantly between euchromatic and heterochromatin parts of the chromosomes. Ramanna and Prakken (1967) made a comparison of the mean lengths of these chromatin segments between pachytene chromosomes and the corresponding metaphase chromosomes and found that proximal heterochromatic segments were reduced in length by a factor of 4-5, whereas the euchromatic parts might differ by a factor of 25-30. This implies that such differential contraction should be taken into account when deducing physical distances between markers on the chromosomal DNA. In addition, the more condensed chromatin segments might also be less accessible to probes in FISH experiments.

FISH conditions for both metaphase and pachytene spreads have been established using three different types of repetitive DNA sequences, two of which with known chromosomal location, ribosomal DNA and the telomere-associated repeat TGR1. (Ganal *et al.*, 1988; Lapitan *et al.*, 1989). In all cases studied, FISH on pachytene spreads was clearly superior in providing signals at a much higher resolution. As for the dispersed repeat probe THG2, for example, FISH on pachytene spreads showed a highly defined dispersion pattern with signals mainly confined to the heterochromatin regions and large stretches of chromosomes devoid of any signal. In contrast, virtually no differentiation in signal distribution was obtained in employing metaphase spreads, indicating that any future high resolution mapping in tomato should be conducted with chromosomes at meiotic pachytene stage.

As is already common practice in animal and human cytogenetics, FISH offers the possibility to simultaneously visualise multiple targets in a single specimen. In tomato, multicolour FISH would be a powerful tool in constructing integrated chromosome maps. In this regard, the simultaneous detection of two classes of repeats as described in the present paper, provides a first step towards this goal.

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Chapter 3

HIGH-RESOLUTION MAPPING ON PACHYTENE CHROMOSOMES AND EXTENDED DNA FIBRES BY FLUORESCENCE *IN SITU* HYBRIDISATION

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Abstract

This paper describes two protocols for high resolution physical mapping of DNA sequences in tomato using fluorescence *in situ* hybridisation (FISH). The first technique involves FISH to spread chromosomes from pollen mother cells at pachytene and proves to be an excellent method for assigning DNA sequences to chromosome regions at a resolution of up to a few hundred kilobases. An even higher resolution was obtained for extended DNA fibres, prepared from interphase nuclei and used as hybridising component. This technique permits strong enhancement of physical map resolution to values of a few kilobases. The power of both methods simultaneously applied to the same material was demonstrated with the combination of the telomeric repeat and the tomato specific telomere-associated repeat TGR1 as example.

Key words: Fluorescence *in situ* hybridisation, FISH, pachytene chromosomes, extended DNA fibres, tomato.

Abbreviations: DAPI, Diamidino-phenyl-indole; EDTA, ethylene diamine tetraacetate; FISH, fluorescence *in* situ hybridisation; FITC, Fluorescein isothiocyanate; SDS, sodium dodecyl sulphate; SSC, standard saline citrate; TGR, tomato genomic repeat.

Introduction

Multi-colour fluorescence *in situ* hybridisation (FISH) has been developed as one of the major cytogenetic tools for constructing accurate physical maps of single copy markers and repetitive sequences along chromosomes (reviewed by Jiang and Gill, 1994; Joos *et al.*, 1994). Its resolution for human mitotic metaphase chromosomes, usually in the range of 1 to 3 Mb (Lawrence *et al.*, 1990; Lichter *et al.*, 1990), is limited by the high degree of chromatin condensation. Similar results with FISH mapping to metaphase chromosomes of plants, especially in the case of species with relatively small genomes, such as *Arabidopsis*, rice, tomato and sorghum, stimulated the development of protocols for enhanced resolution FISH to interphase and prophase chromosomes or decondensed chromatin.

Nuclei at different mitotic and meiotic stages, including meiotic prophase I, have been compared for their suitability for high resolution mapping. Especially good results have been obtained with chromosomes of pollen mother cells at late pachytene, containing well-spread bivalents generally ten to fifteen times longer than their metaphase counterparts, exhibiting clearly differentiated patterns of light euchromatin and dark

heterochromatin blocks, and thin, weakly stained centromeres. As one of the best cytogenetic model plant species, tomato shows a distinct pachytene karyotype with chromomere patterns unique for each chromosome. This provides cytological maps with a differentiation level close to that of mammalian G-banding, allowing identification of all twelve chromosome pairs in the complement (Ramanna and Prakken, 1967) and providing an outstanding diagnostic tool for high resolution FISH mapping in this species (Zhong *et al.*, 1996).

Improvements in resolution have been achieved with FISH to mammalian interphase nuclei (Lawrence *et al.*, 1990; Trask *et al.*, 1991) and free chromatin or extended DNA fibres (Heng *et al.*, 1992; Haaf and Ward, 1994; Wiegant *et al.*, 1992; Parra and Windle 1993; Heiskanen *et al.*, 1994; Florijn, *et al.*, 1995). Hybridisation of probes to extended DNA fibres allows the delineation and ordering of contiguous sequences at a resolution of a few kilobases (kb) or less (Parra and Windle, 1993; Florijn, *et al.*, 1995). Recently, this powerful technique has been applied in higher plants in order to visualise and map cosmid, lambda and plasmid clones on extended DNA fibres from *Arabidopsis thaliana* and tomato (Fransz *et al.*, 1996).

In this report we present the comprehensive FISH protocols for both pachytene chromosomes and extended DNA fibres of *Lycopersicon esculentum*. Probes of the telomeric repeat [TTTAGGG] from *Arabidopsis thaliana* and the tomato specific telomere-associated repeat TGR1 serve as an example in two-colour FISH experiments for demonstrating the enhancement of mapping resolution of both techniques.

Materials and Methods

Plant material

Lycopersicon esculentum cv VFNT Cherry was used for this study.

Solutions required

Carnoys fluid: 3: 1 ethanol : acetic acid

Pectolytic enzyme mixture: 0.3% cellulase "Onozuka" RS (Yakult Honsha Co., Ltd, Tokyo, Japan), 0.3% pectolyase Y23 (Sigma P3026), 0.3% cytohelicase (Sepracor, France) in 30 mM citrate buffer pH 4.5

Nuclei isolation buffer: 10 mM Tris-HCl pH 9.5, 10 mM EDTA, 100 mM KCl, 0.5 M sucrose, 4.0 mM spermidine, 1.0 mM spermine, 0.1% mercapto-ethanol

PBS buffer: 10 mM sodium phosphate, pH 7.0, 140 mM NaCl

STE nuclei lysis buffer: 0.5% SDS, 5 mM EDTA, and 100 mM Tris-HCl pH 7.0

Hybridisation mixture: 20 µl per slide, containing 50% (v/v) formamide, 2 × SSC, 10% (w/v) sodium dextran sulphate, 50 mM phosphate buffer pH 7.0, 1-2 ng/µl labelled probe DNA, and 50-100 ng/µl salmon sperm DNA

2 × SSC: 0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0

4M buffer: 5% non-fat dry milk in 4 × SSC

4T buffer: 4 × SSC, 0.05% Tween 20

TNT buffer: 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween 20

TNB buffer: 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% blocking reagent supplied by Boehringer Mannheim

Avidin-Texas red: Boehringer Mannheim

Goat-anti-avidin-biotin: Boehringer Mannheim

Sheep-anti-dig-FITC: Boehringer Mannheim

Rabbit-anti-sheep-FITC: Boehringer Mannheim

DAPI: 2 µg/ml in Vectachield antifade

Pachytene chromosomes

Preparations of pachytene chromosomes were made from young anthers containing pollen mother cells at meiotic prophase I.

- Collect flower buds with appropriate length[1].
- Remove one anther from a flower bud using a dissecting microscope, squash in 1% acetocarmine (in 45% acetic acid) and check its stage of development using a phase contrast microscope.
- Transfer the remaining anthers in that flower bud to Carnoy's fluid and fix for 20 minutes at room temperature.
- Wash three times in deionised water.
- For cell wall digestion, incubate the anthers in 1 ml of pectolytic enzyme mixture at 37°C for 2 hours.
- Wash carefully in deionised water and store the vulnerable anthers on ice until use.
- Transfer a single anther to a clean, grease-free slide. Add 1 µl of water and squeeze out its pollen sacs out of the anther with fine needles. Remove as much of the anther tissue as possible.
- Add 5 µl of 60% (v/v) acetic acid to the cell suspension, mix with a needle, and keep it for one minute to clear up cytoplasm.
- Gently put about 1 ml of freshly prepared ice-cold ethanol-acetic acid (3:1) on the microscopic slide, in a circle around the suspension. While extending across the glass surface the fixative will precipitate the cells and stick them to the glass surface. Add few more drops to the cells just before complete evaporation.
- Immerse the wet slide briefly in 96% ethanol, then air-dry thoroughly.
- Check the preparations under the phase contrast microscope and select the best of them for the *in situ* hybridisation experiments.
- The slides can be stored at -20 °C for months.

Extended DNA fibres

We used nuclei from young leaves for making the preparations of extended DNA fibres.

- Collect 2 grams of fresh tomato leaves.
- Freeze the leaves in liquid nitrogen immediately.
- Grind to a fine powder in liquid nitrogen with a precooled mortar and pestle.
- Transfer the powder to a 50 ml centrifuge tube with a precooled spoon.
- Add 20 ml of ice-cold nuclei isolation buffer and mix gently for 5 minutes.
- Filter the homogenate sequentially through one layer each of 170, 125, 50, and 20 μm nylon mesh filters. Keep on ice all the time.
- Add 1 ml of nuclei isolation buffer containing 10% (v/v) Triton X-100 to the filtrate.
- Centrifuge at 2000 × *g* for 10 minutes at 4 °C.
- Resuspend the pellets in 200 μl of nuclei isolation buffer to obtain a final concentration of about 5 x 10^6 nuclei/ml.
- Check quality and concentration of the nuclei suspension by staining a 1 μl sample with DAPI under the fluorescence microscope[2].
- Nuclei stored in glycerol should be washed in PBS buffer by spinning down at 3000 rpm for 5 min in an Eppendorf centrifuge and resuspending in PBS.
- Put 1 μl of nuclei at one end of a clean slide, gently spread the suspension with a pipette tip in a short stroke parallel to the short rim of the microscopic slide and let air-dry for a few minutes.
- Add 10 μl of STE lysis buffer to the nuclei and incubate for 4 minutes.
- Gently tilt the slide with the drop at the upper end to about 45°, carefully moving the drop of buffer across the glass surface, thereby pulling out stretched fibres of DNA into a long stream. Allow the drop to stream toward the other end of the slide and air-dry.
- Fix the DNA fibres with Carnoy's fluid for 2 minutes, air dry and bake the slide at 60 °C for 30 minutes.
- The preparations can directly be used for standard fluorescence *in situ* hybridisation without pre-treatment with RNase, pepsin and formaldehyde.

Probe labelling

Clone pAtT4 (Richards and Ausubel, 1988) containing the telomeric repeat (TTTAGGG) of *Arabidopsis thaliana* was used as probe to detect the tomato telomeric repeat. The tomato specific repeat, TGR1, containing a 162 bp motive (Ganal et al., 1988), was used as probe for the telomere-associated repeat. pAtT4 was labelled with biotin-16-dUTP (Boehringer) and TGR1 with digoxigenin-11-dUTP (Boehringer) by either standard nick translation or random primer labelling according to the instruction of the manufacturer.

Fluorescence *in situ* hybridisation

Pre-treatment of preparation with RNase, pepsin and formaldehyde

The steps below are only required for pachytene chromosomes.

- Incubate preparations in 2 × SSC for 5 minutes.
- Add 100 µl of 100 µg/ml RNase A in 2 × SSC on each slide, cover with a 24 × 50 mm coverslip, and incubate at 37 °C for 1 hour.
- Wash the slides in 2 × SSC three times for 5 minutes.
- Incubate the slides in 0.01 M HCl for 2 minutes.
- Add 100 µl of 5 µg/ml (20 units/ml) pepsin in 0.01 M HCl on each slide, cover with a coverslip, and incubate at 37 °C for 7 to10 minutes.
- Wash the slides in water for 2 minutes and in 2 × SSC two times for 5 minutes.
- Incubate the slides in 1% formaldehyde in PBS buffer pH 7.0 for 10 minutes.
- Wash the slides three times in 2 × SSC for 5 minutes.
- Dehydrate the slides in 70%, 90% and 100% ethanol for 3 minutes each, and let air-dry.

In situ hybridisation and post washes

- Prepare the hybridisation mixture (20 µl per slide)[3].
- Use 20 µl of the hybridisation mixture per slide, and cover it with a 24 × 32 mm coverslip.
- Denature chromosome and probe DNAs at 80°C for 2 minutes.
- Place the slides in a pre-warmed humid chamber and incubate at 37°C for 12-18 hours.
- Prewarm freshly prepared 50% (v/v) formamide / 2 × SSC pH 7.0 to 42°C, and wash the slides in this solution three times for 5 minutes.
- Wash the slides briefly in 2 × SSC at room temperature.
- Rinse the slides in 0.1 × SSC at 56-60°C three times for 5 minutes[4].
- Wash the slides in 2 × SSC for 5 minutes.

Detection and amplification

- Wash the slides in 4T buffer for 5 minutes.
- Add 100 µl of 4M buffer to each slide, cover with a 24 × 50 mm coverslip, and incubate at 37°C for 30 minutes.
- Wash the slides briefly in 4T.
- Add 100 µl of 2 µg/ml Avidin-Texas Red in 4M buffer to each slide, cover with a coverslip, and incubate at 37°C for 60 minutes.
- Wash the slides in 4T buffer for 5 minutes.
- Wash the slides two times in TNT buffer for 5 minutes.
- Add 100 µl of 10 µg/ml goat-anti-avidin-biotin and 10 µg/ml sheep-anti-dig-FITC in TNB buffer to each slide, cover with a coverslip, and incubate at 37°C for 60 minutes.

- Wash the slides in TNT buffer three times for 5 minutes.
- Add 100 µl of 2 µg/ml Avidin-Texas Red and 10 µg/ml rabbit-anti-sheep-FITC in TNB buffer to each slide, cover with a coverslip, and incubate at 37°C for 60 minutes.
- Wash the slides in TNT buffer three times for 5 minutes.
- Dehydrate the slides in 70%, 90%, and 100% (v/v) ethanol.
- Counterstain with 10 µl of 5 µg/ml DAPI in Vectashield antifade to each slide, cover with a 24 × 50 mm coverslip.
- Study the results under a fluorescence microscope with the filter sets for DAPI, FITC and Texas red, respectively.
- Make photographs on a 400 ISO colour negative film.

Notes

1) For the VFNT cherry cultivar, a 3- to 4-mm flower bud corresponds to the pachytene stage.

2) The nuclei can be stored at -20°C for months after mixing with an equal volume of glycerol.

3) In applying FISH to extended DNA fibres, add 10-100 pg/µl probe DNA.

4) The only stringent washing.

Results and Discussion

The protocol for the preparation of pachytene chromosomes was optimised for better spreading of pachytene chromosomes and to minimise background of the pollen mother cell cytoplasm. As revealed by DAPI staining, the distinct differentiated pattern of eu- and heterochromatin was retained, showing prominent fluorescent blocks in the proximal heterochromatin regions and most of the telomeres, and faint fluorescence in both euchromatic parts and the centromeres (Fig. 1a). This fluorescence pattern was found to correspond precisely with that of classical aceto carmine staining (Ramanna and Prakken, 1967). The distinct morphology thus permitted identification of all twelve chromosomes following the criteria for the tomato pachytene karyotype of Ramanna and Prakken (1967).

To evaluate the resolution of FISH to pachytene chromosomes, two adjacent repetitive DNA sequences located at the tomato telomere region were studied in a double labelling hybridisation experiment. Former studies on the macrostructure of the tomato telomere (Ganal *et al.*, 1991) revealed that the tomato telomeres are composed of two repetitive families, *i.e.* the common telomeric repeat and a telomere-associated repeat (TGR1). The telomeric repeat in tomato has a 7-base-pair unit, TT(T/A)AGGG, which easily cross-hybridises to the telomeric repeat of *Arabidopsis thaliana* (TTTAGGG) (Ganal *et al.*, 1991). The telomere-associated repeat TGR1 is a tomato-specific tandem repeat with a 162 bp motive, located on 20 out of the 24 telomeres, and physically adjacent or close to

the telomeric repeat (Ganal *et al.*, 1988; Schweizer *et al.*, 1988; Ganal *et al.*, 1991). A biotin labelled telomeric repeat probe (pAtT4) from *Arabidopsis thaliana* and a digoxigenin labelled telomere-associated repeat probe (TGR1) from tomato were used for simultaneous FISH to pachytene chromosomes (Fig. 1a). After detection and amplification, hybridisation signals of the pAtT4 and TGR1 probes appeared as red and green fluorescent spots, respectively. The pAtT4 probe hybridised to all 24 telomeres, whereas the TGR1 signals could be detected in only 20 of them. These 20 telomeres with a combination of pAtT4 and TGR1 signals could be classified into three groups according to their hybridisation patterns:

(1) the green signals completely separated from the red signals;

(2) the green signals partly overlapping the red signals and therefore showing a pattern of contiguous green-yellow-red spots;

(3) the green signals entirely overlapping the red signals giving rise to uniformly fluorescing yellow spots.

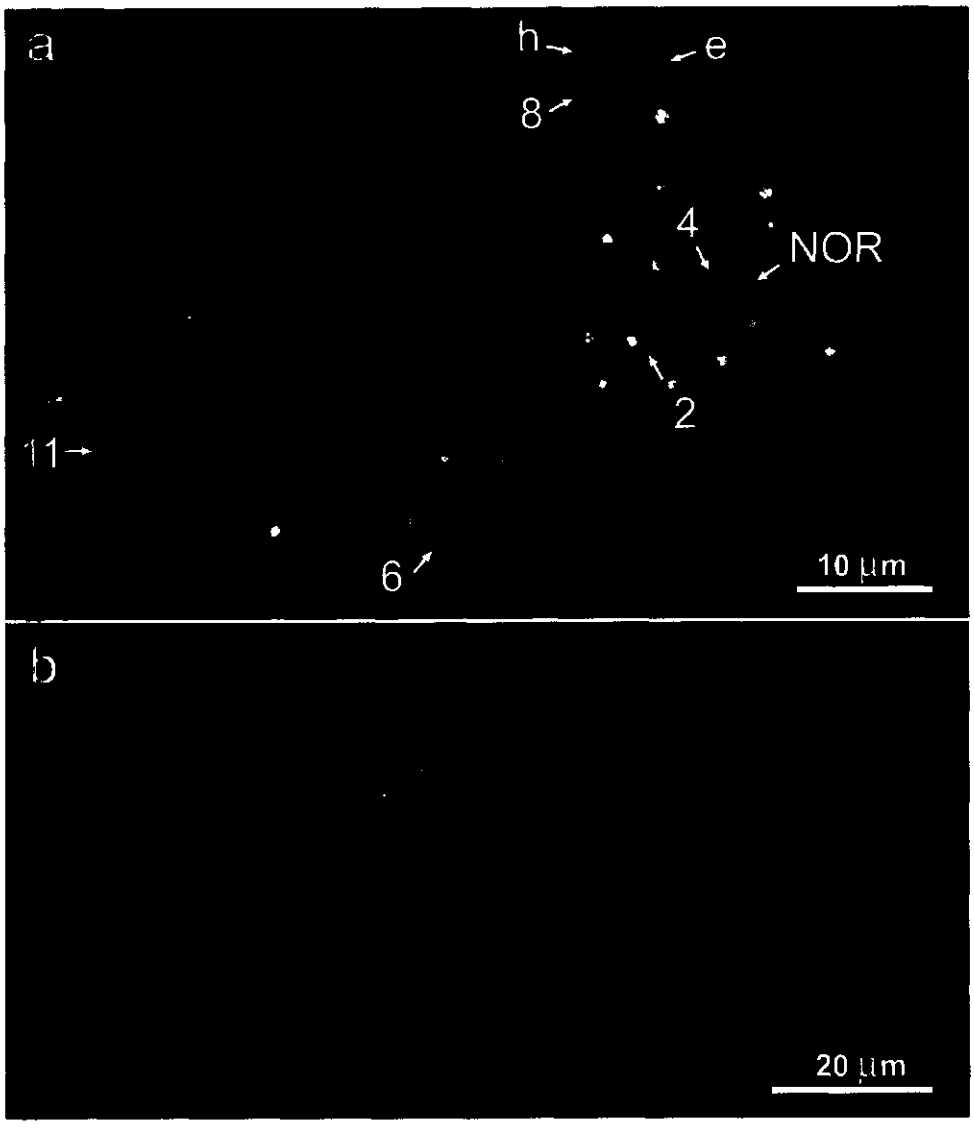
Previous molecular analysis of these telomeres using restriction enzyme digestion, pulsed field gel electrophoresis, and Southern blot analysis (Lapitan *et al.*, 1989; Ganal *et al.*, 1991) have demonstrated that the different TGR1 fragments and the telomeric repeat are physically separated by spacers varying from a few kilobase to several hundreds of kilobases. Based on the three FISH patterns mentioned above, the first group of two telomeres exhibiting completely separated red and green signals will represent chromosome ends with relative long spacers between the telomere and TGR1 regions, while the third group of four telomeres with completely yellow signals has short spacers or no spacer at all.

Chromosomes at pachytene are, on the average, fifteen times longer than at mitotic metaphase, their contraction rate still differing significantly between euchromatic and heterochromatic areas. Ramanna and Prakken (1967), who compared mean proximal and distal segments values for all chromosomes, found that the distal euchromatic areas differ by a factor of 25-30, whereas heterochromatic segments around the centromeres were reduced only by a factor of 4-5. This implies that differential contraction rates should be taken into account for deducing physical distances between markers in both heterochromatin and euchromatin as well as when comparing with corresponding physical distances in mitotic chromosomes, and that physical resolution in euchromatic regions is correspondingly higher than in heterochromatic parts. In addition, very compact parts of the chromosomes, especially in constitutive heterochromatic (C band positive) bands might be less accessible for probes in a FISH experiment. Irrespective of these limitations, the most important advantage of the tomato pachytene chromosomes is the karyotype, based on chromosome specific chromomere patterns, differences in centromere position and relative length, which permit unequivocal assignment of cytogenetic markers including repetitive and unique DNA sequences to the tomato chromosomes. Examples of identified chromosomes are given in the pachytene complement of figure 1a

Unsurpassed resolution in mapping was obtained with FISH to extended DNA fibre. Its characteristic image shows hybridisation signals of fluorescent tracks containing many minor beads rather than the single spots as in pachytene or metaphase chromosomes. Figure 1b shows an example of the FISH of the telomeric repeat and the telomere-associated repeat TGR1 to the extended DNA fibres of two similar chromosome ends. The length of the red fluorescent tracks representing the telomeric repeat was measured at 7.6 mm. Based on a stretching degree of 3.27 kb/mm (Fransz *et al.*, 1996), its corresponding molecular size was estimated at 25 kb, which fits well in the range for length estimates based on molecular data (Ganal *et al.*, 1991). Similar data were obtained for establishing lengths of the spacers and the TGR1 repeats (unpublished results).

Despite its mapping resolution of approx. 1 kb and the detection of target DNA sites smaller then 700 bp (Fransz et al., 1996), FISH to extended fibres does not allow large scale localisation of sequences on the native chromosomes due to the relative short uninterrupted fluorescent traces without any diagnostic chromosomal characteristic like centromeres or C-bands. This limitation, however, is met by similar observations at the pachytene chromosomes. The simultaneous use of both FISH techniques are strongly complementary and gives the opportunity to map sequences on well differentiated chromosomes at a resolution hitherto restricted to molecular biological analyses and will therefore supplement existing tools for molecular cytogenetic analysis of genome organisation, physical mapping and positional cloning in higher plants.

Figure 1. Two-colour fluorescence *in situ* hybridisation of the tomato telomeric repeat (red) and the telomere-associated repeat TGR1 (green) to pachytene chromosomes and extended DNA fibres. The telomeric repeat probe pAtT4 was labelled with biotin-16-dUTP, and detected with Avidin-Texas red. The telomere-associated repeat probe TGR1 was labelled with digoxigenin-11-dUTP, and detected with sheep-anti-DIG conjugated FITC. **a.** FISH to the pachytene chromosomes. NOR indicates the position of the Nuclear Organiser Region of the satellite chromosome, whereas h and e refer to examples of heterochromatic and euchromatic regions, respectively. The arrows with number point to the position of centromeres of their corresponding chromosomes. **b.** FISH to the extended DNA fibres of two similar chromosome ends.


a
h
e
8
4
NOR
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11
6
10 µm
b
20 µm

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Chapter 4

FISH STUDIES REVEAL THE MOLECULAR AND CHROMOSOMAL ORGANISATION OF INDIVIDUAL TELOMERE DOMAINS IN TOMATO

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Abstract

The molecular and cytological organisation of the telomeric repeat (TR) and the subtelomeric repeat (TGR1) of tomato were investigated by fluorescence *in situ* hybridisation (FISH) techniques. Hybridisation signals on extended DNA fibres, visualised as linear fluorescent arrays representing individual telomeres, unequivocally demonstrated the molecular co-linear arrangement of both repeats. The majority of the telomeres consists of a TR and a TGR1 region separated by a spacer. Microscopic measurements of the TR and TGR1 signals revealed high variation in length of both repeats, with maximum sizes of 223 and 1330 kb, respectively. A total of 27 different combinations of TR and TGR1 was detected, suggesting that all chromosome ends have their own unique telomere organisation. The fluorescent tracks on the extended DNA fibres were subdivided into four classes: (i) TR - spacer - TGR1; (ii) TR - TGR1; (iii) only TR; (iv) only TGR1. FISH to pachytene chromosomes enabled some of the TR/TGR1 groups to be assigned to specific chromosome ends and to interstitial regions. These signals also provided evidence for a reversed order of the TR and TGR1 sites at the native chromosome ends, suggesting a backfolding telomere structure with the TGR1 repeats occupying the most terminal position of the chromosomes. The FISH signals on diakinesis chromosomes revealed that distal euchromatin areas and flanking telomeric heterochromatin remain highly decondensed around the chiasmata in the euchromatic chromosome areas. The rationale for the occurrence and distribution of the TR and TGR1 repeats on the tomato chromosomes are discussed.

Introduction

Telomeres have long been regarded as rather static elements of eukaryote chromosomes. Over the past decade, however, a vast body of evidence has accumulated showing that telomeres are actually highly dynamic chromosomal structures associated with key aspects of chromosomal functions and behaviour. Not only do they permit stable replication of chromosome termini (Blackburn, 1994), but they also prevent chromosome disintegration and participate in regulating cell division and cellular senescence (Wright and Shay, 1992). Furthermore, through their association with the nuclear envelope in meiotic prophase I, telomeres are likely to play a critical role in defining nuclear organisation and meiotic pairing (Dernburg *et al.*, 1995).

The telomere structure is composed of a complex of telomere-specific DNA sequences and various proteins (Zakian, 1995). In most species, these sequences involve a

telomeric repeat (TR) consisting of a highly conserved tandem repeat array of a 6 to 8 base pairs (bp) motif at the extreme end of the chromosomes (Biessmann and Mason, 1994). The telomeric repeat is often associated with other tandemly repeated sequences of higher complexity, referred to as the subtelomeric repeats (Ashikawa *et al.*, 1994; Brandes *et al.*, 1995; Ganal *et al.*, 1991; Kilian and Kleinhofs, 1992; Kolchinsky and Gresshoff, 1994; Roder *et al.*, 1993; Vershinin *et al.*, 1995; Wu and Tanksley 1993). These repeats are often species-specific and vary in length and degree of repetitiveness (Biessmann and Mason, 1994).

In tomato, the TR consists of an array of the tandemly repeated oligonucleotide TT(T/A)AGGG sequence that varies in length at different chromosomal ends (Ganal *et al.*, 1991). This repeat is separated by a spacer region of a few hundred kilobases (kb) from a 162 bp satellite repeat, the subtelomeric repeat TGR1 that is found at 20 out of the 24 telomeres (Lapitan *et al.*, 1989; Schweizer *et al.*, 1988). Together, TR and TGR1 sequences account for approximately 2% of the tomato genome (Ganal *et al.*, 1991).

In plants, the molecular organisation and size estimates of telomere DNA sequences have been mostly established by means of pulsed field gel electrophoresis (PFGE) (Brandes *et al.*, 1995; Ganal *et al.*, 1991; Roder *et al.*, 1993; Vershinin *et al.*, 1995; Wu and Tanksley 1993). As to tomato, this technique also permitted the genetic mapping of four telomeres to specific chromosomal arms, exploiting distinct length polymorphisms at the telomeric TGR1 loci and their linkage to the most distal RFLP markers from the saturated RFLP linkage map (Ganal *et al.*, 1992). However, there are limitations in analysing the molecular organisation of telomeres by PFGE. Firstly, molecular sizes are usually overestimated due to the positions of restriction sites for rare-cutting enzymes close to but not at the proximal border of the repeats. Secondly, PFGE provides size estimates of repeat domains representing average figures for the entire genomic DNA, but is not suited for assigning specific repeats to individual chromosomes and for analysing length heterogeneity at individual chromosomal loci.

The aforementioned limitations can be overcome by the combined application of fluorescence *in situ* hybridisation (FISH) to both extended DNA fibres and chromosomes. On the one hand, fibre FISH provides an effective tool to study the molecular organisation of individual telomere repeats enabling their direct physical mapping on individual DNA strands. This technique has been shown to be suitable for spanning several hundreds of kilobases at a resolution to the extent of at least a few kilobases (Fransz *et al.*, 1996; Parra and Windle, 1993; Wiegant *et al.*, 1992). On the other hand, FISH to pachytene chromosomes provides supplementary information on the mapping of repeat domains to specific chromosomal regions (Xu and Earle, 1996a and b; Zhong *et al.*, 1996a).

Here we address the question of how individual TR-TGR1 domains are organised at individual chromosome ends in terms of length heterogeneity, spacer distribution, sequence

composition and compactness. By applying FISH to extended DNA fibres we were able to visualise the different types of TR-TGR1 organisation among individual chromosome ends. Similarly, FISH to pachytene and diakinesis chromosomes revealed new aspects of the telomeric domains at higher order chromatin organisation.

Results

Molecular organisation of the TR and TGR1 repeats as revealed by PFGE and FISH on extended DNA fibres

In order to examine the long range organisation of the telomere repeat (TR) and the subtelomeric repeat TGR1, at first PFGE was applied. To this end, genomic DNA was cut with *BglII* or *EcoRV*, restriction enzymes that are both known to leave the two repeats unimpaired (Ganal *et al.*, 1991). The resulting restriction fragments were resolved by PFGE under conditions that allow resolution up to 2000 kb, blotted and hybridised to TR and TGR1 probes (Fig. 1). TR-containing fragments were found to range in size between 50 and 2000 kb with the bulk appearing in the 50 - 250 kb size range. In both the *BglII* and *EcoRV* digests, some discrete bands of *c.* 700 kb, 880 kb and 1900 kb were apparent.

TGR1 fragments ranged in size from 100 to 2000 kb. The bulk of fragments, with sizes between 300 - 800 kb, was clearly larger than the TR-containing fragments. Again, some discrete large-sized fragments were observed, some of which co-migrated with the TR fragments, indicating co-linearity and the absence of a *BglII* or *EcoRV* site within the telomere domain.

In an attempt to dissect the molecular organisation of the TR and TGR1 repeats at individual DNA molecules, extended genomic DNA fibres (Fransz *et al*, 1996) were subjected to dual-label FISH using the biotinylated pAtT4 clone containing the TR of *Arabidopsis thaliana* and digoxigenin-labelled TGR1 from tomato as probes. Thus, a fluorescent pattern of parallel running linear tracks of red and green spots was obtained, representing individual chromosomal regions consisting of TR and TGR1 sequences, respectively (Fig. 2a). All signals showed continuous dotted tracks, suggesting that both repeat units were tandemly reiterated without interruption by other sequences, though the presence of intervening sequences spanning a few kilobases cannot be excluded. The orientation of TR and TGR1 relative to the position of the residual nuclei (arrows at the bottom of Fig. 2a) clearly showed the order of the repeats, the telomere repeat was distal to the subtelomeric TGR1. Most of the patterns consisted of red and green signals separated by a non-fluorescent spacer. Occasionally red or green signals were observed that lacked any association with the other repeat.

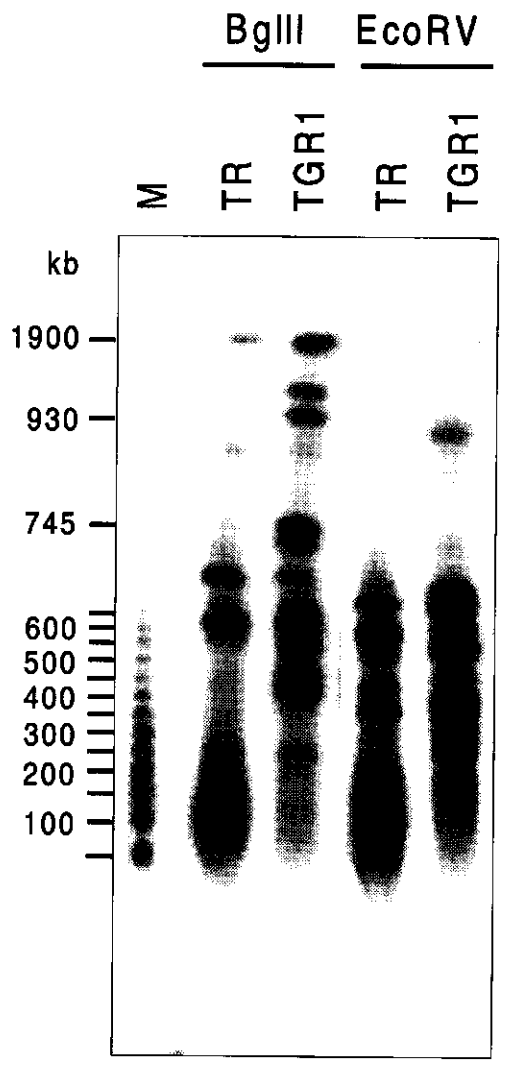


Figure 1. Southern blot analysis of tomato telomeres. High molecular weight DNA was digested with BglII and EcoRV, separated by PFGE, blotted and hybridised with the telomeric repeat TR and the subtelomeric repeat TGR1.

To determine the microscopic lengths of the TR-TGR1 regions, we selected 80 clearly distinguishable, individual patterns (Fig. 2b-e) and measured both the length of the green and red fluorescing tracks and the non-fluorescent spacer. For conversion of the microscopic length (μm) into a molecular size (kb), a stretching degree of 3.27 kb μm^{-1} was applied, a value that has been derived from similar FISH experiments with known DNA sequences on tomato and *Arabidopsis* extended DNA fibres (Fransz *et al.*, 1996).

In order to classify the FISH patterns into groups that might correspond to individual chromosomal sites, we sorted them according to decreasing lengths of spacer, telomere and TGR1 (Table 1). The order of sorting keys is based on the fact that the spacer image is not affected by fibre breakage, while the images of the short telomere and especially the long TGR1 arrays are. The patterns were grouped on the basis of length similarity of the TR-spacer-TGR1 combination. To reduce misinterpretation of the signals, at least two of the three regions should have a minimum and maximum value that deviate less than five percent of the average. Should there be any doubt as to the assignment of fluorescent signals to certain groups, the length of the non-fluorescent spacer gave conclusive evidence.

The classification resulted in 26 groups, each consisting of two or more fibres with identical or nearly identical fluorescence patterns, and five groups containing single fibres with a unique pattern. Four of these (numbers 11, 24, 25 and 26 in Table 1) were most probably individual examples of broken fibres with incomplete TR and TGR1 signals, and could be assigned to any of the 26 groups with more than one fibre. However, the one with the longest TGR1 signal and the largest spacer (number 31 in Table 1) was probably the only representative of this independent group, giving a total of 27 groups. These were further subdivided into 4 classes based on the presence of the telomere signal, the TGR1 signal and the spacer: class I, a TR array associated with a TGR1 array via a non-fluorescent spacer (Fig. 2b); class II, a TR array directly associated with TGR1 (Fig. 2c); class III, a TR array, without TGR1 (Fig. 2d); class IV, a TGR1 array, without TR (Fig. 2e).

The 27 groups most probably correspond to the 24 telomeric ends plus three interstitial TGR1 sites (class IV). Although conclusive evidence is lacking, such a direct relationship is reinforced by the fact that the number of groups with only a TGR1 signal (class IV) matches the number of interstitial TGR1 sites, while the number of groups without a TGR1 signal (class III) corresponds to the number of chromosomal ends lacking TGR1 (see below).

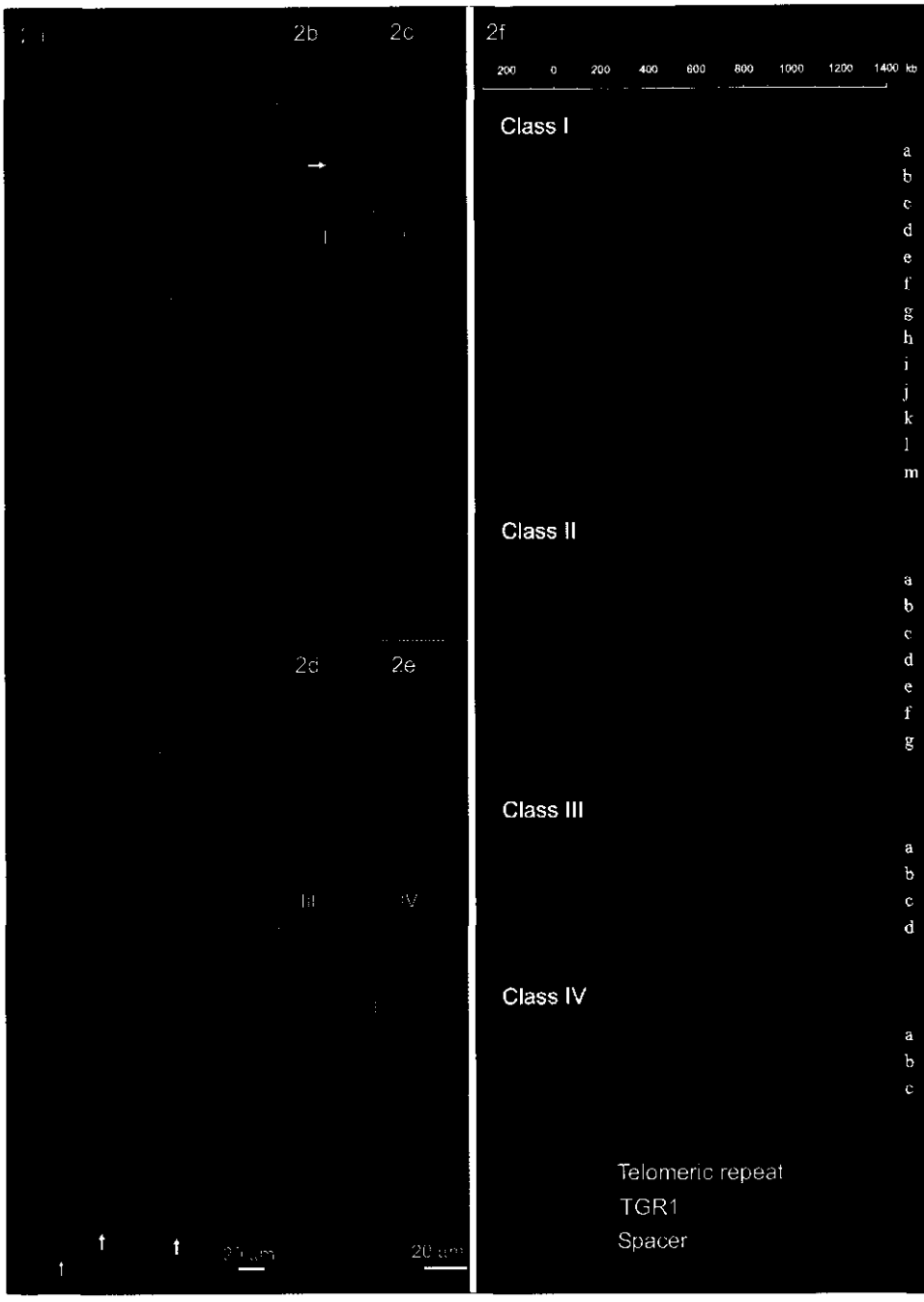
Figure 2. Two-colour FISH of the tomato telomeric repeat (red) and the subtelomeric repeat TGR1 (green) to extended DNA fibres. **2a.** An overview of long hybridisation signals in a large area of the microscopic slide (200 x 10 000 μm^2) composed of 13 partially overlapping photographs. The arrows indicate the position of the residual nuclei. **2b.** Class I: TR in association with the TGR1 repeat, but separated by a spacer (arrows). **2c.** Class II: TR directly flanking to TGR1 without a spacer. **2d.** Class III: TR free of any TGR1 sequence. **2e.** class IV: TGR1 free of any TR at interstitial sites. **2f.** A diagram of different TR-TGR1 combinations from 80 individual fibre signals. Similar signals are grouped to be considered from identical or homologous chromosomal regions. Bar = 20 μm.

Table 1. Overview of the average molecular sizes in kb of the TR, TGR1 and the spacer regions from 80 individual FISH patterns, sorted in sequence of decreasing length for spacer, TR and TGR1.

pattern	n	TR		spacer		TGR1		group*)
		average	min-max value	average	min-max value	average	min-max value	
1	3	0	0-0	0	0-0	88.3	85-95	IV-a
2	4	0	0-0	0	0-0	157.0	137-170	IV-b
3	4	0	0-0	0	0-0	634.3	585-703	IV-c
4	3	25.0	23-26	0	0-0	0	0-0	III-a
5	3	44.7	39-49	0	0-0	0	0-0	III-b
6	4	67.0	59-72	0	0-0	0	0-0	III-c
7	2	121.0	118-124	0	0-0	0	0-0	III-d
8	2	15.5	13-18	0	0-0	404.5	402-407	II-c
9	3	26.7	26-27	0	0-0	217.7	210-224	II-b
10	2	38.0	36-40	0	0-0	402.5	402-403	II-d
11	1	67.0	-	0	-	457.0	-	-
12	3	74.3	72-76	0	0-0	143.7	143-144	II-a
13	4	93.3	90-98	0	0-0	736.5	716-761	II-g
14	4	117.0	111-121	0	0-0	513.8	495-543	II-f
15	2	160.0	160-160	0	0-0	363.0	360-366	II-e
16	2	77.0	72-82	13.0	13-13	395.5	389-402	I-d
17	2	46.0	46-46	17.0	16-18	233.5	228-239	I-a
18	6	85.2	82-92	20.5	16-23	493.0	474-502	I-f
19	3	125.3	121-130	17.3	16-18	523.3	497-537	I-g
20	2	216.0	209-223	17.0	16-18	307.0	301-313	I-e
21	2	101.0	101-101	22.0	22-22	197.0	184-210	I-b
22	2	107.0	107-107	22.0	22-22	319.5	317-322	I-c
23	2	157.0	157-157	24.5	22-27	730.0	721-739	I-j
24	1	101	-	31	-	180	-	-
25	1	98	-	36	-	529	-	-
26	1	56	-	40	-	611	-	-
27	3	100.0	98-101	43.3	40-46	853.7	850-860	I-k
28	4	115.0	111-121	49.3	45-54	600.8	592-609	I-i
29	2	49.0	49-49	73.0	71-75	934.0	887-981	I-l
30	2	108.5	101-116	75.5	75-76	492.0	487-497	I-h
31	1	134.0	-	98.0	-	1330.0	-	I-m

n, number of observations

*) The group number corresponds to the classification shown in Figure 2f.


2b
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Class IV
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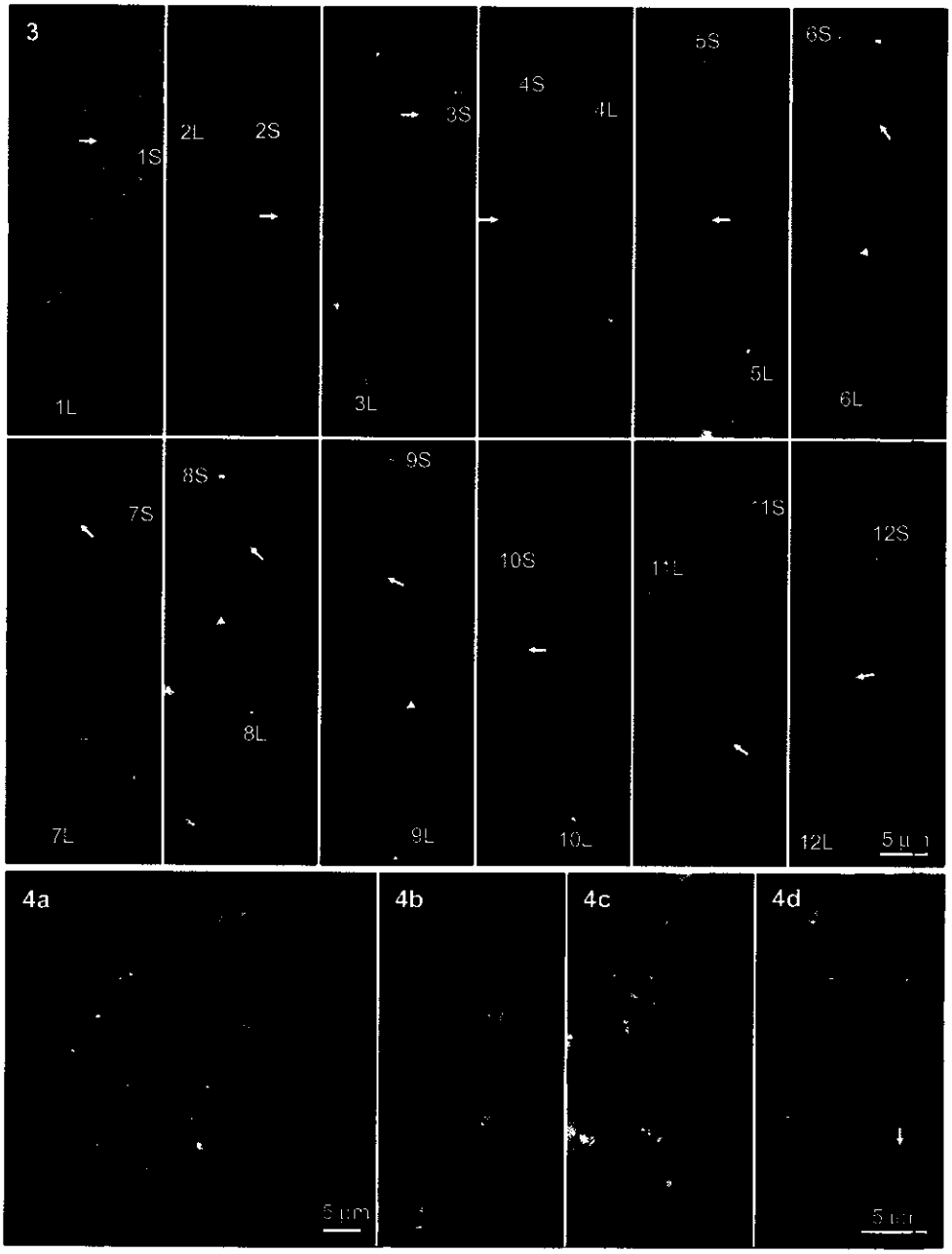
Telomeric repeat
TGR1
Spacer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12L
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4a
5 µm
4b
4c
4d
5 µm

The length of the regions containing the telomeric repeat varied from 13 to 223 kb with an average of 88.6 kb. The TGR1 tracks spanned 85 - 1330 kb with an average of 465.8 kb, while the length of the spacer ranged from 13 to 98 kb, with an average of 34.3 kb. Regression analyses for all combinations of TR, spacer and TRG1 lengths revealed a significant correlation only for the lengths of the spacer and TGR1 ($r = 0.69$, $P < 0.01$), whereas all other combinations of TR, spacer and TGR1 proved to be not or weakly correlated.

The telomere lengths for the class III chromosome arms (1L, 2S, 2L and 7L, see below) ranged from 23 to 124 kb, with an average of 59.9 kb, but they could not be assigned to individual chromosomes. Class IV most likely represents interstitial TGR1 sites that are distributed among three distinct groups with molecular sizes of 85 - 95 kb, 137 - 170 kb and 585 - 703 kb, respectively.

Position and organisation of the TR and TGR1 on pachytene and diakinesis chromosomes

We next probed the TR and TGR1 repeats on pachytene and diakinesis chromosomes in order to localise their position on each individual chromosome arm and to study their cytological organisation on chromosomes with different condensation patterns. Pachytene chromosomes were chosen for this type of analysis as they are relatively easy to identify (see Fig. 3) (Ramanna and Prakken, 1967; Zhong et al., 1996b) and superior to metaphase chromosomes (Ganal *et al.*, 1991; Lapitan *et al.*, 1989) in subchromosomal mapping. The red fluorescent TR signals were detected at all 24 chromosome ends, while TGR1 (green) hybridised to 20 ends. The three long arms 1L, 2L, 7L and one short arm 2S, which harbours the nuclear organizer region (NOR), were devoid of any terminal TGR1 signal, an observation that corresponds with the class III groups (see above) representing the TR signals on DNA fibres that are not associated with TGR1.

Figure 3. Two-colour FISH of the tomato telomeric repeat (red) and the subtelomeric repeat TGR1 (green) to pachytene chromosomes. The numbers with *S* and *L* indicate short and long arms, respectively, of corresponding chromosomes. The arrows point to the positions of centromeres. The arrow heads indicate the interstitial sites of TGR1. Bar = 5 μm.

Figure 4. Two-colour FISH of the tomato telomeric repeat (red) and the subtelomeric repeat TGR1 (green) to diakinesis chromosomes. **4a.** A complete cell with 12 bivalents. **4b.** Two bivalents with distal chiasmata showing hybridisation signals on highly decondensed chromatin. **4c.** Two bivalents with interstitial chiasmata showing the signals on decondensed chromatin. **4d.** Two bivalents showing signals in condensed telomeric chromatin of the arms lacking chiasmata. Note the decondensed chromatin in the subtelomeric region of the opposite arm (arrow). Bar = 5 μm.

In addition to the telomere-linked signals, three interstitial TGR1 signals were detected in the middle of the chromosome arms 6L, 8L and 9L, respectively. Again the pachytene data were in accordance with the fibre FISH data showing the three groups of class IV. By using a monosomic addition of tomato chromosome 6 in a potato background, we could assign the 157 kb TGR1 signal of group IVb to the interstitial site of 6L (Zhong *et al.*, manuscript in preparation). From the FISH results, an ideogram was constructed (Fig. 5) showing the chromosomal localisation of the TR and TGR1 for each chromosome.

All TGR1 and TR signals, distal as well as interstitial, co-localised with heterochromatic chromomeres, suggesting that the repeats occur in highly condensed areas of chromatin. The occurrence of a red-yellow-green pattern indicates that the resolution of the condensed telomeric region in pachytene is lower than the distance between the distal telomere end and the proximal TGR1 end, which can be as large as 200 kb. Surprisingly, most of the telomeric TGR1 signals were, unlike their location on fibres, observed at the very ends of the pachytene chromosomes, distal from the TR signals (Fig. 3), indicating a reversed order of TR and TGR1.

Shortly after pachytene, the chromatin undergoes dramatic conformational changes. While the homologues start disjoining, chromosomes rapidly de-condense forming a fine network of thin threads. During this transient diffuse diplotene stage, individual chromosomes are temporarily indiscernible, with their heterochromatin regions appearing as clustered indistinct structures (Cawood and Jones, 1980). Shortly later, just before entering diakinesis, chromosomes start a second process of condensation, initially at the centromere heterochromatic regions only. The homologous distal euchromatin segments, however, which remain associated through their chiasmata until anaphase I, retain their decondensed status throughout diakinesis.

DAPI staining revealed bright fluorescence of the centromeric heterochromatin areas of the diakinesis bivalents, which was in contrast to the distal regions around the putative positions of the chiasmata showing weak or no DAPI staining at all. However, the TR and TGR1 signals were clearly resolved, visualising the decondensed state of these regions with striking variation in intensity and pattern among the bivalents (Fig. 4a-d). We distinguished three different patterns: (i) highly decondensed chromatin with long, non-overlapping stretches of TR and TGR1 signals (Fig. 4b); (ii) decondensed chromatin with separate, single spots of TR and TGR1 (Fig. 4c); and (iii) highly condensed chromatin with partly overlapping TR and TGR1 signals (Fig. 4d).

Each pattern could be explained on the basis of differences in chiasma position of the euchromatin arm regions. In the first case we assume a distal chiasma, very close to the telomeres. The entire distal region, including the telomeric heterochromatin, will remain despiralised, resulting in non-overlapping extended TR and TGR1 signals. The second type will occur in those cases where chiasmata occur in the interstitial regions. As a result the

distal euchromatin and telomeric blocks will be relatively condensed and, hence, keep the TR and TGR1 closer together as solid, partly overlapping structures. In the third type, which occurs in the unbound arms of rod bivalents, euchromatin becomes highly condensed, showing compact telomere regions with small overlapping TR/TGR1 signals (Table 2).

Table 2. The relationship of chromatin condensation in the telomere regions and the positions of chiasmata in diakinesis bivalents

Position of chiasmata in a chromosome arm	(n)	Percentage of chromosome arms with distal chromatin condensation		
		highly decondensed	decondensed	condensed
distal	(98)	57.1	40.8	2.0
interstitial	(53)	15.1	66.0	18.9
no chiasma	(41)	-	22.0	78.0

Discussion

Molecular organisation and pachytene mapping of the telomeric repeats

In this paper we present detailed maps of the telomeric and subtelomeric repeat arrays of all tomato chromosome arms, exploiting the extended DNA fibre technique as primary tool. The expected organisation of telomeres containing domains for the telomeric repeat, spacer and subtelomeric repeat has now been visualised directly under the fluorescence microscope as distinct combinations of bicolour fluorescent arrays, in specific cases separated by a short non-fluorescent spacer region. Previously, we have shown that the extended DNA fibres of tomato and *A. thaliana* hybridised to various types of probes, including repeats (5S rDNA), cosmids, λ-clones and plasmids, and provide accurate molecular maps of various chromosomal regions that are in accordance with maps established by conventional molecular techniques (Fransz *et al*, 1996; Fransz *et al*, 1998; Zhong *et al*, 1996b). Unlike PFGE mapping, however, the fibre FISH technique has the advantage in providing information regarding the organisation of repeat domains on individual DNA molecules. Similar to the length measurements of the 5S rDNA repeat

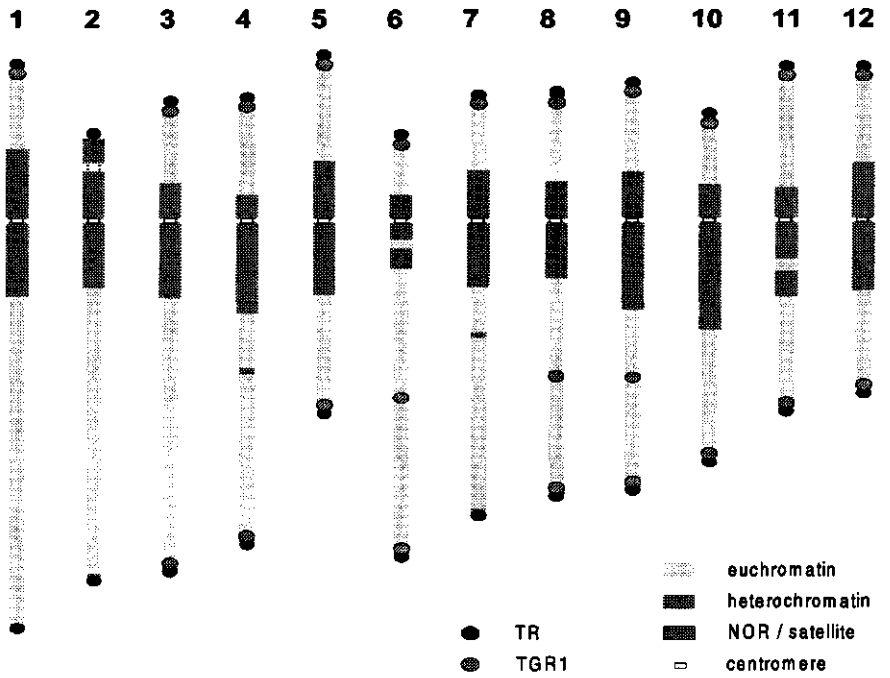

Figure 5. Ideogram of the telomeric repeat and TGR1 sequences on pachytene chromosomes composed on the basis of the FISH results.

array in tomato (Fransz *et al*; 1996; Lapitan *et al*, 1991), size estimates of the tomato TR determined by PFGE and fibre FISH were in accordance with the bulk of the TR domains, ranging in size between 50 and 250 kb and between 13 and 233 kb, respectively. Likewise, no major differences were found regarding the size distribution of the TR-TGR1 domains as determined by the respective techniques.

As to the TR repeats, our length measurements put the tomato telomeric domains in the upper size range found for other plant telomeric repeats, which vary from 3.5 kb in *Arabidopsis* (Richards *et al*, 1992) to 30 kb in rice (Wu and Tanksley, 1993). As our FISH experiments with the telomeric repeat hybridised to extended genomic DNA fibres from *Petunia*, maize, potato, rice and *Arabidopsis* confirm the corresponding published length measurements based on PFGE (unpublished data), the relative large TR arrays found for tomato are unlikely to be an artifact. It may thus be argued that the length estimate of the tomato TR domain (30 - 60 kb), as determined by combined *Bal31/HaeIII* digestion (Ganal *et al*, 1991) is an underestimation, probably due to cryptic *HaeIII* sites present within the long array.

Twenty three different groups for the subtelomeric and interstitial TGR1 repeat, varying from 85 to 1330 kb, were found. This number is slightly less than the 30 groups in Lapitan's PFGE analysis with *BglII*-digested tomato DNA showing fragments ranging from 25 to 1000 kb (Lapitan *et al.*, 1989). The same argument as discussed above (presence of cryptic restriction sites) for the TR discrepancy may again account for the inconsistency with respect to the minimum TGR1 size, though it cannot be excluded that small TGR1 fragments (*c.* 25 kb) that were considered as background in the DNA fibre preparations remained undetected in the pachytene complements.

The occurrence of a specific TR and a specific TGR1, in combination or alone, and with or without the non-fluorescent spacer revealed 31 TR - TGR1 groups, encompassing the 24 chromosomal ends and the three interstitial TGR1 sites. The number of TR - (spacer -) TGR1 combinations amounted to 24 (class I and II), slightly more than the 20 chromosome ends with both repeats as observed in pachytene complements. It is plausible that a few of the TR - TGR1 combinations became slightly longer or shorter at their borders due to some technical artefacts, and so were erroneously classified as different groups. Even with these technical artefacts that may cause some fibre length variation, our data strongly suggest that most, if not all, chromosome ends have their own unique telomere organisation with dissimilar lengths for TR, spacer and TGR1.

Although their function is not clear, it is speculated that the TGR1 domains and the spacer sequence might play a role as buffering blocks separating chromosome ends from unique sequences. Alternatively, subtelomeric repeats have been suggested to mediate chromosome fusion and fission in vertebrates (Meyne *et al.*, 1990). Subtelomeric tandem repeat sequences have been cloned from onion (Barnes *et al.*, 1985), rice (Wu *et al.*, 1991) and barley (Brandes *et al.*, 1995), while three non-homologous, tandemly repeated DNA families were present in subtelomeric regions of rye (Vershinin *et al.*, 1995). In addition, telomere associated sequences (TAS) have been detected in barley (Kilian and Kleinhofs, 1992), maize (Burr *et al.*, 1992) and soybean (Kolchinsky and Gresshoff, 1994). It seems fair to speculate that the telomere-associated repeats comprise a complex mixture of repetitive sequences composed of a number of different repeats, specially for species with large genome sizes. The surprising result that the spacer has a minimum value of 13 kb and that its length increases proportionally with TGR1 lengths suggests that the spacer contains repeat elements and that spacer and TGR1 are co-evolving and in some way functionally related.

Non-telomeric sites for TR and TGR1

FISH with the *Arabidopsis* TR probe to pachytene chromosomes revealed clear signals on all tomato telomeres, but none on interstitial or centromere sites. TR repeat sequences at interstitial locations have been reported in many vertebrate (Meyne *et al.*, 1990) and plant species (Alfenito and Birchler 1993; Richards *et al.*, 1991) and are considered as remnants of chromosome rearrangements that occurred during genome evolution. In addition, Presting *et al.* (1996) have detected many telomere homologous sequences near the centromeres in tomato chromosomes. Such interstitial TR repeat sites are speculated to be the result of centromere-telomere recombination and ancient chromosome fusion or arm inversions (Presting *et al.*, 1996). Comparison of the tomato genome with those of closely related taxa provided evidence for five arm inversions from potato (Tanksley *et al.*, 1992), and 15 inversions and translocations from pepper (Tanksley *et al.*, 1988; Prince *et al.*, 1993). This indicates that the tomato genome has undergone many chromosome rearrangements, giving rise to interstitial TR sites near centromeres. The absence of FISH signals from TR sequences near centromeres may reflect either a number of TR units too small to be detected by FISH or a change in the TR sequence originating from the telomere. Sequence analysis of some interstitial TR fragments indeed revealed a degenerate or interspersed pattern of TR units (Presting *et al.*, 1996).

The occurrence of TGR1 seems to be more dynamic in terms of karyotype evolution and its distribution pattern is probably different from that of the telomere repeats. Rather than assuming chromosomal rearrangements, as might be true for TR, we postulate that telomere distribution is largely determined by the spatial relationship of chromosomes in the interphase or prophase nuclei. During these stages chromosomes retain their anaphase arrangement, showing centromeres and telomeres facing opposite poles, also known as the Rabl orientation. This situation brings centromeres and telomeres in proximity, enabling DNA sequences to jump from a chromosome to a non-homologue. Evidence has been reported in the mouse (Garagna *et al.* 1993) of the distribution of similar satellite repeats over non-homologous chromosomes by a jumping mechanism during the formation of the bouquet polarisation at prophase I of meiotic cells. The attractiveness of this assumption is that it also explains the occurrence of equidistantly located interstitial TGR1 sites as being copied from short arm sites, including the 3S, 4S, 8S, 9S and 10S arms. This phenomenon, referred to as 'equilocal' distribution (Heitz, 1932), has been described for many species with conspicuous heterochromatic knobs or C-bands (*e.g.* Greilhuber and Loidl, 1983). The distribution assumes a mechanistic model, in which the repeats are distributed to other chromosomal loci nearby in the spatially ordered nucleus (Bennett, 1982).

The number of interstitial TGR1 sites detected in our study (6L, 8L and 9L) is lower than the six sites observed by Lapitan *et al.* (1989) in primary tomato trisomics. This

difference presumably reflects polymorphism differentiating the two genotypes. In addition, in assuming that all interstitial TGR1 sites co-localise with the diagnostic chromomere in carmine-squashed pachytene chromosomes of the tomato cultivars Moneymaker, Glory-m and Artresist (Ramanna and Prakken, 1967), the chromosome arms 3L, 4L, 7L and 12L are likely to contain similar TGR1 sites.

Higher order organisation of the chromosome ends

During meiosis and mitosis, chromosomes pass through a process of extensive chromatin condensation, which reaches a maximum rate during the metaphase stage. Using telomere-specific probes, FISH studies have provided valuable information regarding the higher order organisation of the chromosomal ends. It has been reported that the TR in mouse pachytene cells is exclusively associated with the two ends of each autosomal synaptonemal complex (SC), the axial core structure to which chromatin loops are attached (Moens and Pearlman, 1990a). Furthermore, by applying FISH to pachytene cells and extended DNA fibres of the mouse, Heng *et al.* (1996) showed that different chromatin packaging mechanisms exist for interstitial vs. terminal chromosomal regions, independent of the DNA sequences. As the TRs in tomato are mapped at the extreme chromosome ends, distal from the TGR1 repeats, we would expect a similar order in condensed pachytene chromosomes. The observed order, however, appeared to be reversed, suggesting a crosier-like foldback structure of the telomere region. Similar observations of subterminal TR signals have been reported in pachytene chromosomes of mouse (Moens and Pearlman, 1990b) and metaphase chromosomes of human (Moyzis *et al.*, 1988), grasshopper (Suja and Rufas, 1994), pea and field bean (Rawlins *et al.*, 1991) and several other dicots (Cox *et al.*, 1993).

It has been suggested, that the chromatin loops of the subtelomeric region encompass the telomeric end (Moens and Pearlman, 1990a; Suja and Rufas, 1994). However, if the TGR1 chromatin loops would spread around and past the telomeric end, only a green and a yellow signal would be expected as a result of TGR1 overlapping the entire TR, which was not the case. Based on these resolvable TR and TGR1 signals, green-yellow-red, a backfolding structure of the telomeres would involve the entire TR region, the spacer and at least part of the TGR1 region, which could span up to 1000 kb. Although the Carnoy fixation of the pachytene cells generates a more compact appearance of chromosomes, thereby affecting the spreading of the chromatin loops, it does not affect the order of the signals.

The subterminal position of the telomeric end is suggested to be related with protection of the telomeres from exonucleolytic degradation, fusion and recombination. The

reversed order implies that in condensed pachytene chromosomes the subtelomeric repeat region is the extreme end, suggesting that they might play a role not only in telomere protection, but also in the attachment of interphase and prophase chromosomes to the nuclear membrane. This may shed a new light on the as yet unknown function of subtelomeric satellite repeats.

FISH to diakinesis chromosomes revealed the majority of chiasmata to be located at distal euchromatin segments of the chromosome. Without the FISH technique the distal chiasmata would not have been observed due to the fact that the region flanking the distal chiasma is highly decondensed and, hence, not visible with DAPI staining alone. This may explain why distal chiasmata have remained so far refractory to detection, although their occurrence can be predicted on the basis of late recombination nodule distributions that occur in tomato along all euchromatin segments and not in proximal heterochromatin or very distal ends (Sherman and Stack, 1995), the latter propably being the regions containing the TR - TGR1. The highly decondensed structure, which covers hundreds of kilobases up to more than 1 Mb, supports previous data on decondensed chromatin surrounding chiasmata. It is generally accepted that the idea of terminalisation of chiasmata is not valid. Hence, the distal chiasmata represent cross-overs close to or within the telomeric region. The latter might explain the hypervariability at the telomeric ends, as found in the TR-TGR1 domain of tomato (Broun *et al.*, 1992) and other species, which is supposed to be the result of either mutation or unequal cross-over.

The data presented in this paper clearly demonstrate that FISH techniques are a very valuable tool, not only to map DNA repeats to chromosomes, but also to elucidate the molecular and chromosomal organisation of tandemly repeated DNA sequences of the telomeric domain.

Experimental procedures

Plant Material and chromosome preparation

Lycopersicon esculentum cv VFNT Cherry (2n = 24) was used in all experiments. Chromosome spreads at pachytene and diakinesis were prepared from young anthers containing pollen mother cells at meiotic prophase I, as described previously (Zhong *et al.*, 1996b). Preparations of extended DNA fibres were made from young leaf material according to the protocols of Fransz *et al.* (1996, 1997) and Zhong *et al.* (1996b).

DNA probes

The clone pAtT4 (Richards and Ausubel, 1988) containing the telomeric repeat TTTAGGG of *A. thaliana* was used as a probe to detect the tomato TR sequence. A clone containing a 162 bp repeat motif of

tomato satellite repeat TGR1 (Schweizer *et al.*, 1988) was used as a probe for the tomato subtelomeric repeat sequence. pAtT4 and TGR1 were labelled with biotin-16-dUTP (Boehringer) and digoxigenin-11-dUTP (Boehringer), respectively, using either standard nick translation or random primer labelling according to the instruction of the manufacturer.

Pulsed field gel electrophoresis (PFGE)

High molecular weight (> 2 Mb) genomic tomato DNA was isolated from young leaves according to Van Daelen and Zabel (1994) and digested with *BglII* or *EcoRV* (Ganal *et al.*, 1991). Restriction fragments in the range 50 - 2000 kb were resolved on a CHEF gel (electrophoresis conditions: 60 sec pulses for 16 hours followed by 90 sec pulses for 7 h at 6 V cm^{-1}) . Southern blotting and hybridisation were performed as described elsewhere (Van Daelen and Zabel, 1994) using $\alpha[^{32}P]$dATP-labelled probes.

Fluorescence *in situ* hybridisation

Multi-colour FISH of the telomeric repeat and the subtelomeric repeat were performed according to the protocols described by Zhong *et al.* (1996b). The hybridisation signals were visualised under a Zeiss fluorescence microscope with separate excitation filter sets for DAPI, FITC and Texas red. The three-colour images were photographed by triple exposure on 400 ISO colour films. Selected negatives were scanned at a resolution of 1000 d.p.i. and their images were optimised for best contrast and brightness using the image processing software Photoshop (Adobe Inc.).

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Chapter 5

A NOVEL STRATEGY FOR ESTABLISHING THE MOLECULAR ORGANISATION OF SPECIFIC REPEAT SITES USING HIGH RESOLUTION FISH TO ALIEN CHROMOSOMES OF TOMATO-POTATO MONOSOMIC ADDITIONS

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Abstract

The molecular organisation of the telomeric repeat TR and the subtelomeric repeat TGR1 of tomato chromosome 6 was studied in a potato background. Genomic *in situ* hybridisation revealed the presence of a single tomato chromosome among 48 potato chromosomes in a monosomic addition, derived from a somatic hybrid after two backcrosses. RFLP analysis using fourteen molecular markers, spanning the whole linkage map of tomato chromosome 6, revealed full integrity of the alien chromosome. However, supplementary GISH and FISH studies on somatic and pachytene complements showed that the distal end of the long arm, beyond the most distal RFLP markers, was replaced by a piece of potato chromatin as a result of translocation or homoeologous recombination in the somatic hybrid or BC1 plant. Fibre FISH analysis confirmed the existence of only two tracks of TGR1 signals in the monosomic addition, *i.e.* one in combination with a contiguous TR track, representing the distal end of the short arm, and a second TGR1 signal, without TR, that is likely to represent the long arm interstitial TGR1 site. Fibre FISH also allowed size estimates of the tandem repeats of the TR/TGR1 and TGR1 patterns and the data were compared with molecular data from Southern analysis of high molecular weight DNA separated by PFGE. This resulted in an integrated molecular-FISH map of these repeats. Further analysis of the somatic hybrid and the BC1 plants revealed a drastic reduction in size of subtelomeric TGR1 repeats on tomato chromosomes. The process of DNA repeat changes in hybrid plants is discussed.

Keywords: *FISH, extended DNA fibres, tomato, monosomic addition, telomeric repeat, subtelomeric repeat, PFGE, Southern analysis*

Introduction

Due to their abundance in eukaryotic genomes, repetitive DNA families were among the first sequences to be isolated, cloned and analysed (reviews in Flavel 1980, 1986; Dean and Schmidt, 1995). Since then an increasing number of studies have considerably contributed to our understanding of their organisation, evolutionary relationships and chromosomal localisation. Thus, it has become apparent, that repetitive sequences, although substantially non-coding, either in tandem or dispersed organisation, play a role in nuclear house holding functions, including chromosome movement during cell divisions, meiotic pairing, recombination, and gene regulation.

The highly conserved 45S and 5S rDNA families, and the telomeric repeat comprise a major group of tandem repeats. A second group includes a hypervariable and heterogeneous group of satellite repeats which are unique for one or a few related species and generally occur in long arrays of millions of copies. The latter group is merely confined to subtelomeric and pericentromeric regions and to C-band heterochromatin segments, as shown for wheat (Gerlach *et al.*, 1980), rye (Vershinin *et al.*, 1995); *Arabidopsis* (Martinez-Zapater *et al.*, 1986), tomato (Ganal *et al.*, 1988) and *Beta* (Schmidt and Heslop-Harrison, 1993). Due to their species specificity, tandem repeats provide in most cases reliable landmarks for identifying parental chromosomes in interspecific and intergeneric hybrids. Therefore, they can be used as supplementary tools for analysing chromosome transmission and karyotype organisation in breeding programmes involving interspecific hybridisations and in taxonomic studies of putative allopolyploids.

However, little is known about size and organisation of individual repeat arrays on chromosomes and their position relative to low- and single copy sequences. Although molecular marker techniques have been applied to localise repeats on physical and genetic linkage maps (Ganal *et al.*, 1992), their abundant occurrence in the genome and high copy number has, in general, been a major obstacle in defining the precise position relative to unique sequences that serve as reference points on the genetic linkage maps.

The advent of Pulse Field Gel Electrophoresis (PFGE) systems, like contour-clamped heterogeneous electric field (CHEF) gels (Chu *et al.*, 1986) has made the separation of large DNA fragments possible and the technique is now routinely used for characterisation of YAC and BAC clones (*e.g.* Hwang *et al.*, 1991; Bonnema *et al.*, 1996; Woo *et al.*, 1994; Wang *et al.*, 1995) and for estimating the size of both tandem and interspersed repeats in higher plants (Ganal *et al.*, 1991; Wu and Tanksley 1993; Salentijn *et al.*, 1994). In particular the combination of PFGE with fluorescence *in situ* hybridisation (FISH) has gained importance in establishing size and distribution of specific repeat families as shown for rye (Vershinin *et al.*, 1995), tomato (Ganal *et al.*, 1991) and barley (Röder *et al.*, 1993; Brandes *et al.*, 1995). Nevertheless, a few obstacles still remain: (*i*) the molecular size estimation by PFGE is dependent on the position of the restriction sites; (*ii*) the determination of numbers and molecular sizes of the tandem repeats by FISH on the highly condensed chromosomes is quite inaccurate; (*iii*) no specific information is obtained on size and copy number of tandem or dispersed repeats at specific sites on the chromosomes, should the repeat occur at various positions on different chromosomes.

Recent advances in the fluorescence *in situ* hybridisation technology allow improved detection and resolution of repetitive and single copy DNA sequences in chromosome preparations. Multicolour FISH of different probes hybridised to pachytene chromosomes has further added to the detection of DNA targets smaller than 1 kb and made a resolution of 0.1 – 1.2 Mbp feasible (Zhong *et al.*, 1996). Simultaneous hybridisations to stretched

DNA fibres from chemically disrupted interphase nuclei have further enhanced resolution limits and contiguous signals, only 1 kb apart, were resolved in the fluorescence microscope (Wiegant *et al.*, 1992; Parra and Windle 1993; Fransz *et al.*, 1996).

In tomato three major satellite repeats have been detected: (*i*) TGR1, which occurs in the subtelomeric regions of most chromosome arms as well as on interstitial chromomeres of a few long arms and in some proximal regions, and varies in size from 25 to 1000 kb (Ganal *et al.*, 1988; Lapitan *et al.*, 1989); (*ii*) TGR2 (c. 4200 copies per genome) that is scattered over the chromosomes except for chromosome 2 (Ganal *et al.*, 1988), and (*iii*) TGR3 (c. 2100 copies per genome) that is found in the centromeric regions and at interstitial sites of a few chromosomes, and at dispersed sites along most of the chromosome arms (Ganal *et al.*, 1988). All these molecular analyses involved rough estimates of the repeats and did not provide specific molecular data on particular repeat sites.

In a recent paper that combined high resolution FISH to pachytene chromosomes and extended DNA fibres of tomato, we presented accurate data on size and distribution of the subtelomeric repeat TGR1 and the telomeric repeat (TR). We could discern 27 different combinations of these repeats corresponding to 24 unique chromosome ends and three interstitial sites. The fluorescent tracks on the extended DNA fibres were subdivided into four classes, *viz.* TR-spacer-TGR1, TR-TGR1, only TR and only TGR1. For each of these classes we described 13, 7, 4 and 3 groups of fluorescent tracks, respectively, which were assumed to represent unique sites on the tomato chromosomes. As extended DNA fibres loose their position on native chromosomes, it was not possible to allocate most of the tracks on the chromosome (cytogenetic) map.

Two strategies are feasible for mapping tandem repeat arrays on a chromosome map. Firstly, by allocating repeat arrays relative to single copy sequences with known positions on the genetic and chromosome map in a series of multicolour FISH experiments. This approach is extremely laborious and time consuming and requires a large genomic library of YACs, BACs or cosmids. Secondly, by limiting the number of expected repeat sites to plants containing a single alien chromosome or chromosome segment added to a diploid or tetraploid complement of a related species. These so called monosomic additions and their derived disomics with recombinant chromosomes have been produced upon backcrossing of an interspecific or intergeneric hybrid with one of its parental species for a variety of crop species, including *Beta procumbens* in sugar beet (Van Geyt *et al.*, 1988), *B. webbiana* in sugar beet (Reamon-Ramos and Wricke, 1992), *Oryza australiensis* in rice (Multani *et al.*, 1994), *Brassica albaglabra* in *B. campestris* (Chen *et al.*, 1992, 1994), *Nicotiana* sylvestris in *N.* plumbaginifolia (Suen *et al.*, 1997), B. *oleracea* in *B. campestris* (McGrafth *et al.*, 1990), rye in wheat (Wang *et al.*, 1996), oat in maize (Ananiev *et al.*, 1997), potato in *Lycopersicon peruvianum* (Ramulu *et al.*, 1996) and tomato in potato (Carriga-Calderé *et al.* 1998).

This report presents the analysis of an integrated molecular and cytogenetic analysis of the TR and TGR1 repeats on the alien chromosome of a monosomic chromosome addition. This plant isolated from a second backcross of an allohexaploid somatic tomato (+) potato hybrid was found to possess tomato chromosome 6 in a tetraploid potato background (Garriga-Calderé *et al.*, 1998). We analysed the data using multicolour FISH to mitotic cell complements, pachytene chromosomes and extended DNA fibres of the monosomic addition and its crossing parents, and compared the results with Southern analyses of comparable PFGE data. It is shown that the tomato chromosome in this monosomic addition plant contains only two sites of TGR1, one at the distal end of the short arm and one interstitial site in the long arm, allowing precise measurements of their repeat lengths. Some unexpected results in the PFGE study suggesting decrease of molecular size and copy numbers for the two repeats in the somatic hybrid and the BC1 are discussed.

Materials and methods

Plant materials

The allohexaploid somatic hybrid #3117 of *Solanum tuberosum* (+) *Lycopersicon esculentum* with 2n=6x=72 was backcrossed with tetraploid potato as described elsewhere (Jacobsen et al. 1995; Garriga-Calderé 1997, 1998). The first BC1 plant (#6701) recovered via embryo rescuing was found to contain nine tomato chromosomes in addition to four complements of potato. Supplementary analyses by Southern hybridisation with chromosome specific RFLP markers and genomic *in situ* hybridisation of nuclei at diakinesis revealed two sets of the tomato chromosomes 1, 3 and 6, plus single copies of the chromosome 8, 9 and 10. In the second backcross family we selected one plant (#6731-14) with 49 chromosomes. Genomic painting and RFLP analysis demonstrated that the extra chromosome in this aneuploid was tomato chromosome 6. The schematic representation of the crossing scheme is given in Figure 1. All plants were maintained as *in vitro* cultures and were grown up in the greenhouse for collecting root tips and leaf material.

Probe DNAs

Total genomic DNA of the tomato parent C31, potato parent 1017, the somatic hybrid, and the BC1 and BC2 plants were isolated from young leaf material according to Klein-Lankhorst *et al.*, 1991) and sonicated to 1-12 kb fragments for probe labelling and blotting in genomic *in situ* hybridisation. We selected fourteen different RFLP probes (GP164, TG297, TG232, TG153, TG240, H1A12, TG253, TG162, TG275, TG99, TG215, TG314, TG193 and TG221), known to be polymorphic with respect to the potato genome and to cover the entire molecular genetic map (van Wordragen *et al.*, 1996) including most distal markers for both chromosome arms (Table 1, Fig. 2). Clone pAtT4 (Richards and Ausubel, 1988) containing the telomeric repeat from *Arabidopsis thaliana* was used as a probe to detect the telomeric repeat in tomato. The tomato

specific satellite repeats TGR1 and TGR2 (Schweizer *et al.*, 1988; Ganal *et al.*, 1988) were used to detect tomato chromosomes in the intergeneric hybrid and backcross plants.

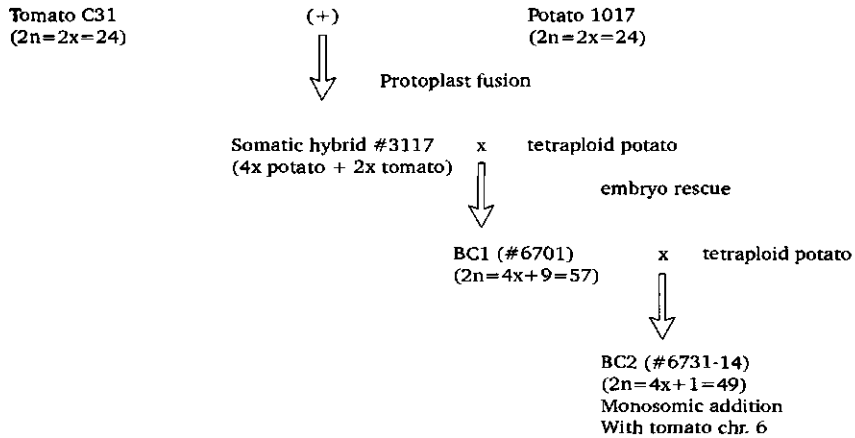


Fig. 1. Breeding programme for the production of a monosomic addition of tomato chromosomes in a tetraploid potato background. Full details were given elsewhere (Jacobsen *et al.* 1995).

RFLP analysis

Probes were labelled with $\alpha[^{32}P]$ATP using the random hexamer method of Feinberg and Vogelstein 1983). The procedures for restriction digestion, electrophoresis, Southern blotting and hybridisation were performed according to Klein-Lankhorst *et al.* (1991).

Pulse Field Gel Electrophoresis (PFGE)

High molecular weight DNAs of at least 2 Mb were isolated from young leaves according to Van Daelen and Zabel (1994) and digested with *BglII* and *EcoRV*, that are known to cut outside the repeats (Ganal *et al.*, 1991). Separation of large DNA fragments in the range from 50 – 2000 kb was performed on a CHEF system (Van Daelen and Zabel, 1994) using a pulsed time of 60 seconds for 16 hours followed by 90 seconds for 7 hours at a voltage of 6.0 V/cm. Southern blotting and hybridisation were carried out as was described for the RFLP analysis.

Table 1. Molecular sizes of the diagnostic RFLP bands in the somatic hybrid, the BC1 and BC2 plants

RFLP markers	Restriction enzyme	ID bands in tomato C31	ID bands in potato 1071
GP164	HaeIII	2.0 kb	0.5 kb
TG25	EcoRI	9.3 kb	2.4 kb
TG99	EcoRI	6.0 kb	2.5 kb
TG162	DraI	1.9 kb	2.7 kb
TG193	BstNI	4.0 kb	2.4 kb
TG215	DraI	1.6 kb	1.0 kb
TG221	BstNI	10.3 kb	5.4 kb
TG232	BstNI	5.5 kb	2.2 kb
TG253	BstNI	6.0 kb	3.6 kb
TG297	HaeIII	6.9 kb	5.4 kb
TG314	EcoRI	12 kb	5.3 kb
TG352	EcoRI	9.3 kb	2.4 kb
TG444	DraI	2.6 kb	5.3 kb
TG548	EcoRI	5.4 kb	4.8 kb

Note: The ID band in the table indicates the specific band sizes for the tomato and potato parents. In case of more than one specific bands present, one band has been selected for listing.

Chromosome preparations and Fluorescence in situ *hybridisation*

Mitotic chromosome preparations were prepared from fast growing root tip meristems and *in vitro* cell cultures. Meiotic chromosome spreads at meiotic prophase were obtained from young anthers as described in a previous paper (Zhong *et al.*, 1996a). Extended DNA fibres were made from chemically disrupted interphase nuclei from young leaves according to the protocols of Fransz *et al.* (1996) and Zhong *et al.* (1996b).

Genomic tomato DNA was labelled with FITC-dUTP following a standard protocol for random primed labelling (Boehringer, Mannheim). Genomic *in situ* hybridisation with total genomic tomato DNA as probe hybridised to chromosomes of the somatic hybrid and backcross products was performed according to Jacobsen *et al.* (1995). The clone pAtT4, TGR1 and TGR2 were labelled with biotin-16-dUTP (Boehringer) or digoxigenin-11-dUTP (Boehringer) either by standard nick translation or by random primer labelling according to the instructions of the manufacturer. Multi-colour FISH of pAtT4 and TGR1 or TGR2 to metaphase chromosomes and extended DNA fibres was carried out according to Zhong *et al.* (1996b). Microphotographs were taken under a Zeiss Axioplan photomicroscope equipped with an epi-fluorescence system and filters for DAPI, FITC and Texas-Red fluorochromes. Selected images on 400 ISO negative colour film were scanned at 500 dpi and digitally processed for optimal brightness and contrast.

Results

The long arm of the alien tomato chromosome 6 lacks distal tomato sequences

Our first goal was to study the integrity of the alien chromosome in the monosomic addition by means of RFLP and chromosomal markers for tomato chromosome 6. In figure 2 a schematic representation of the linkage map of tomato chromosome 6 is given showing the position of the fourteen RFLP markers used for this study (see van Wordragen *et al.*, 1996). The genetic map distance from the most distal markers for both arms, GP164 and TG221 (Fig. 2), amounts to 102 cM (Tanksley *et al.*, 1992). Genomic DNA of tomato C31, potato 1017, somatic hybrids #3117, BC1 #6701 and BC2 #6731-14 were digested with an appropriate restriction enzyme (*EcoR*I, *BstN*I, *Hae*III or *Dra*I) and, after separation by electrophoresis, the DNA fragments were blotted and hybridised with probes of the selected markers. The markers that gave polymorphic bands for the tomato and potato parents, allowed us to establish their presence in the somatic hybrid, and BC1 and BC2 plants. All tomato chromosome 6 bands appeared in the BC2 plant (see Table 1 and examples of Southern hybridisations for GP164, TG253, and TG221 in Fig. 2). The RFLP analysis thus revealed full integrity of the region between the markers of GP164 and TG221 of the alien tomato chromosome.

In a subsequent series of experiments, we focused on the FISH map of the alien chromosome to include the distal repeat families. Firstly, a GISH experiment to mitotic metaphase complements using FITC-dUTP labelled tomato genomic DNA as probe confirmed the presence of a single tomato chromosome in the BC2 plant (Fig. 3a). However, a closer examination at higher magnification made clear that there were no FITC signals at the distal part of the long arm of this chromosome (see arrow in Fig. 3c). A similar GISH experiment to other preparations containing spread pollen mother cells at pachytene revealed a short stretch of propidium iodide (PI) fluorescing chromatin at the distal end of this chromosome (see arrow in Fig. 3d). At this stage, the length of short and long arms of this chromosome amounted to 8.3 µm and 28 µm, respectively. De PI region measured 2 µm which equals 7 % of the long arm. It was assumed that the distal part of the tomato chromosome arm 6L was replaced by a potato chromosome fragment. Additional evidence came from a two-colour FISH with probes for the telomeric repeat (TR) from *Arabidopsis thaliana* and the tomato specific subtelomeric repeat TGR1 hybridised to mitotic metaphase chromosomes. Yellow fluorescing spots appeared at the distal end of the short arm representing the overlapping (red) TR and (green) TGR1 signals and a pair of smaller green fluorescing spots located in the middle of the long arm of the alien chromosome (figs. 3e and 3f). There was no TGR1 signal on the distal end of the long arm as expected for the native tomato chromosome 6 (Zhong *et al.*, 1998).

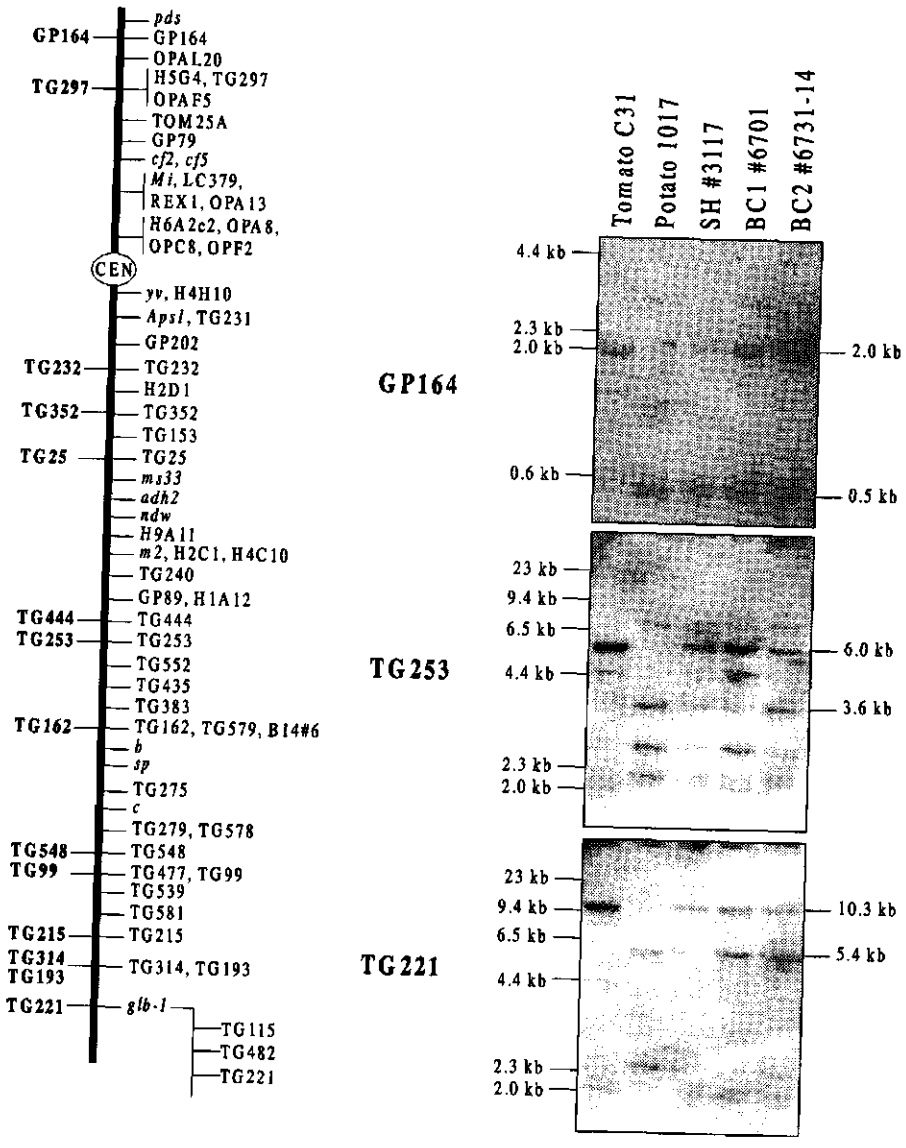


Fig. 2. Integrity of the tomato chromosome 6 in the monosomic addition plant #6731-14 analysed by RFLP markers. Fourteen RFLP markers (at left side of the map), which show polymorphism between tomato and potato, were selected from a molecular map (van Wordragen *et al.*, 1996), including most distal markers from both short and long arm (GP164 and TG221). Genomic DNAs of parents of tomato C31 and potato 1017, somatic hybrid #3117, BC1 #6701 and BC2 #6731-14 were isolated and digested with appropriate restriction enzymes. The DNAs were blotted and hybridised with probes of the selected RFLP markers.

Molecular organisation of the TR and TGR1 repeats at the short arm of the alien tomato chromosome

The results of the aforementioned experiments demonstrated the presence of only two sites of TGR1 on the alien tomato chromosome 6 in the monosomic addition: a larger one at the short arm co-localised with TR and a smaller one in the middle of the long arm. The molecular sizes of the two TGR1 sites were estimated by applying FISH directly to extended DNA fibres obtained from leaf nuclei of the monosomic addition plant. Upon hybridisation with a biotinylated TR probe (red colour) and a digoxigenin-labelled TGR1 probe (green colour), three classes of fluorescent patterns were observed (Fig. 4a and 4b). (1) Red fluorescent tracks without a flanking green signal representing the TR sequences from all potato chromosome ends. The signals vary in size from 2 to 18 μm, which correspond to 7 to 60 kb, respectively, on the basis of a stretching degree of 3.27 kb/μm (Fransz *et al.*, 1996); (2) Red fluorescing TR tracks directly flanked by a long green TGR1 signal (Fig. 4a and 4b) representing the contiguous TR-TGR1 sequences at the short arm of the tomato chromosome 6. The lengths of the signals spanned 5 ± 1 μm for the TR repeat and 125 ± 10 μm for the TGR1 repeat, respectively (Fig. 4c); (3) Green fluorescent tracks figures not connected to any red signals (Fig. 4d). The signals in this class amounted to 50 ± 3 μm (Fig. 4e) and are likely to represent the hybridisation signal of the interstitial site in the long arm. Converting the fluorescent track lengths into molecular sizes gives 16 ± 3 kb for the distal short arm TR, 408 ± 33 kb for the distal short arm TGR1 and 163 ± 10 kb for the interstitial long arm TGR1 site, respectively (Fig. 4c).

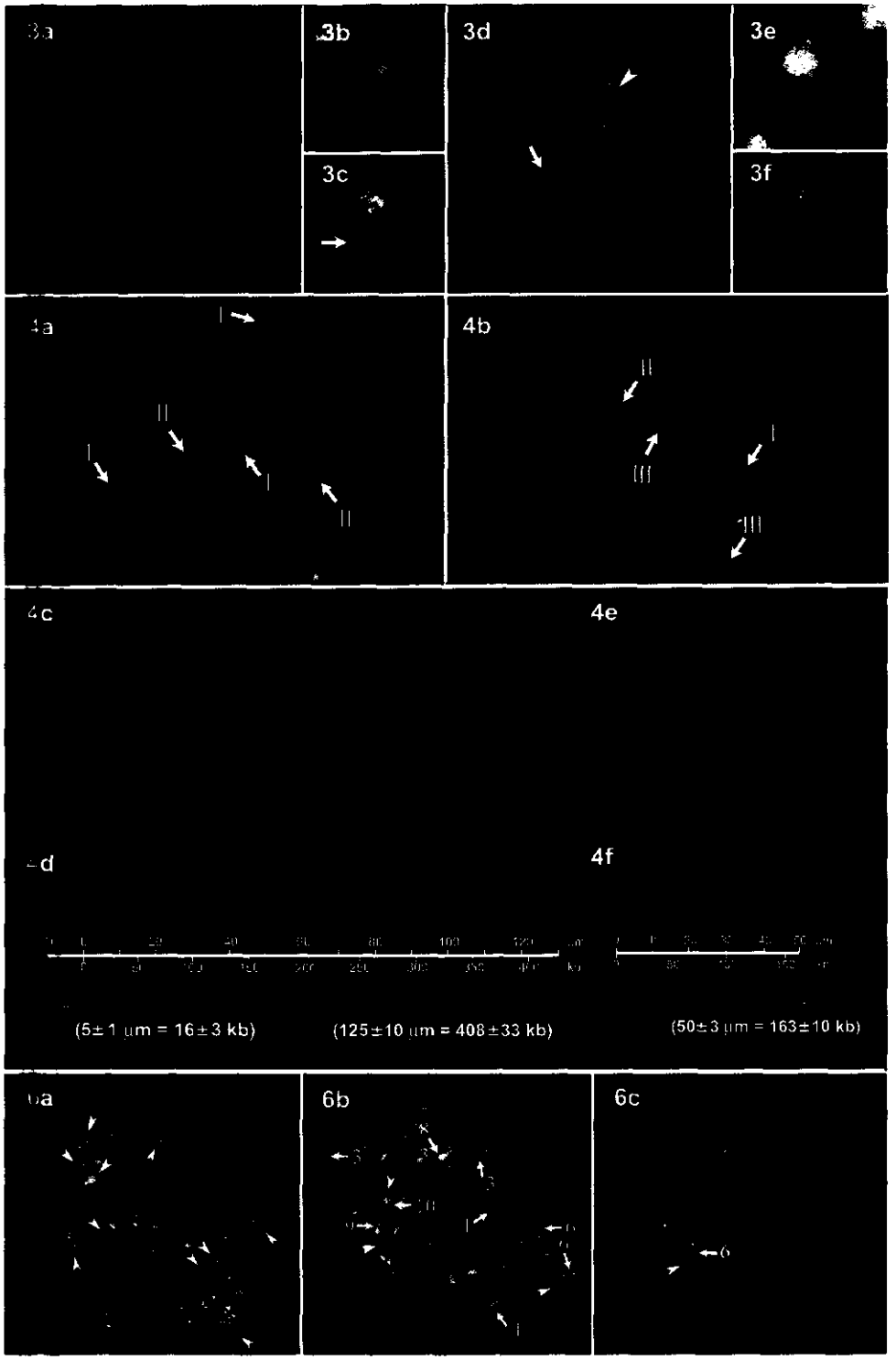
Further analysis of molecular sizes was carried by pulsed field gel electrophoresis (PFGE). High molecular weight DNAs from the tomato and potato parents, the somatic hybrid, the BC1 and the BC2 plants were digested with the restriction enzymes of *Bgl*II and *Eco*RV, which were known to cut outside the TR and TGR1 repeats (Ganal *et al.*, 1991). The DNA fragments were separated by PFGE, blotted and hybridised with a probe containing the TR repeat of *Arabidopsis thaliana* (Fig. 5a). In the potato parent, the majority of TR containing fragments are smaller than 150 kb for both *Bgl*II and *Eco*RV digests. The tomato parent revealed a smear ranging from 50 to 250 kb. In addition, we detected some discrete bands between 600 and 800 kb in both the *Bgl*II and *Eco*RV digests, and two bands of approximately 880 kb and 1900 kb in the *Bgl*II digest. The majority of the TR in the somatic hybrid, and the BC1 and BC2 plants were similar in size to that of the potato parent, but some tomato TR bands with larger sizes were also apparent in those plants. The number of the tomato TR bands gradually decreased through backcrossing, finally resulting in only one extra *Bgl*II band at 560 kb and one extra *Eco*RV band at 470 kb in the monosomic (BC2) plant. This band is assumed to be derived from the tomato chromosome 6, because it was not found in the potato parent.

Figure 3. Cytogenetic analysis of the integrity of the alien tomato chromosome in the potato background by GISH and FISH. **3a.** GISH to the metaphase complements of the monosomic addition using tomato genomic DNA as probe. The chromosome with green fluorescent FITC signals is the alien tomato chromosome. **3b** and **3c.** A close examination of the alien chromosome in DAPI counterstain and FITC signals, respectively. The arrow in **3c** indicates absence of the FITC signals at the distal part of the long arm. **3d.** GISH to pachytene chromosomes of the monosomic addition. The arrow head points to the position of the centromere. The arrow indicates the region with 2 μm (7% of the long arm) chromatin length without FITC signals. **3e** and **3f.** FISH of TR (red) and TGR1 (green) to the alien tomato chromosome. The overlapping TR and TGR1 signals appeared at the distal end of the short arm and a pair of smaller TGR1 signals located in the middle of the long arm. No TGR1 signals were detected at the distal end of the long arm where a pair of the TR signals was located.

Figure 4. Molecular organisation of the TR and TGR1 repeat families in the alien tomato chromosome analysed by Fibre-FISH. **4a** and **4b.** Fibre-FISH signals of TR (red) and TGR1 (green). (I) a red fluorescent TR track without a flanking green TGR1 signal. (II) a short red fluorescent TR track directly flanked by a long green TGR1 signal. (III) a green fluorescent TGR1 track not connected to any red TR signals. **4c.** and **4e.** A number of the Fibre-FISH signals from class II and III was arranged, respectively. The molecular sizes of the TR and TGR1 domains were estimated by the lengths of the FISH signals × the stretching degree of 3.27 kb/μm found previously (Fransz *et al.*, 1996). **4d.** and **4f.** An almost identical Fibre-FISH signal of TR and TGR1, and a TGR1 only, respectively, from original tomato preparations.

Figure 6. Dynamics of the TGR1 domains in the somatic hybrid, and the BC1 and BC2 plants revealed by FISH to metaphase complements. **6a.** the somatic hybrid. **6b.** BC1. **6c.** BC2, the monosomic addition. The tomato chromosomes are identified in red colour by the probe of TGR2 which is tomato specific satellite repeat. The arrow heads point to the distal ends of the chromosomes without TGR1 signals. The chromosomes in BC1 and BC2 are identified by chromosome morphology and TGR1 karyotype.

When the same blot was hybridised with the TGR1 probe (Fig. 5b) only tomato DNA showed signals, thus confirming the specificity of TGR1 for the tomato genome. The majority of the TGR1 fragments in the tomato parent were in the 300 - 800 kb range. Again, some discrete, large sized fragments were observed, some of which co-migrated with the TR fragments, indicating that the two repeat families are collinear without *Bgl*II or *Eco*RV sites between the TR and TGR1 repeats. As to the TR signals, the number of the TGR1 bands were smaller in the somatic hybrid, the BC1 and the BC2, in particular there were fewer bands of large sized DNA fragments. In the BC2 plant, only one *Bgl*II fragment with a molecular size of 560 kb was detected with the TGR1 probe identical in size to the TR *Bgl*II fragment in the same plant. This indicates that both the TR and TGR1 probes hybridised to a *Bgl*II fragment of 560 kb, implying that a *Bgl*II restriction site is present at 560 kb from the physical end of the short arm of the alien tomato chromosome. A darker band of 450 kb and a lighter band of 580 kb were also found in the *Eco*RV digest of the BC2 plant. The former *Eco*RV band corresponds in size with the TR signal, suggesting that a *Eco*RV site is located at 450 kb from the physical end. The 500 kb *Eco*RV band probably reflects the interstitial TGR1 site on the long arm. Differences in band intensity of the two *Eco*RV fragments imply that the darker 450 kb band contains a TGR1 sequence with larger molecular size. This result corresponds well to the length estimates derived from our Fibre-FISH experiment as described in the previous section showing that TGR1 at the telomere of the short arm has a molecular size of 408 kb, while the TGR1 site at the interstitial site on the long arm equals 163 kb.


3a
3b
3c
3d
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4a
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(5±1 µm = 16±3 kb)
(125±10 µm = 408±33 kb)
(50±3 µm = 163±10 kb)
6a
6b
6c

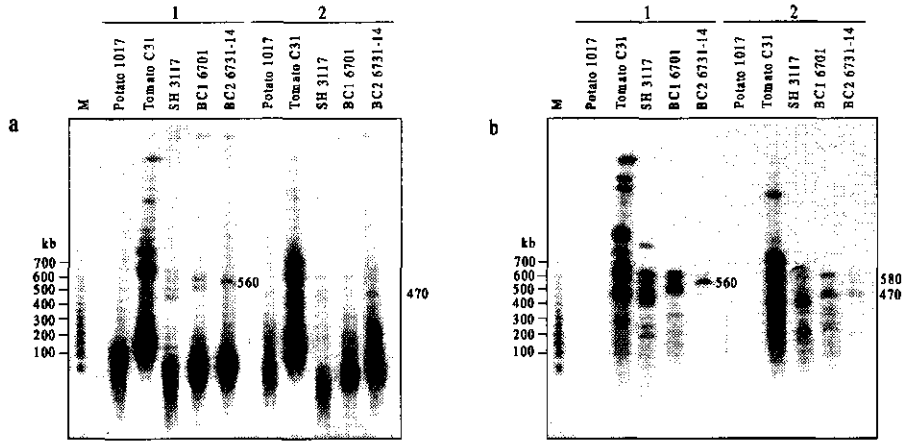

Figure 5. Pulsed field gel electrophoresis analysis. High molecular weight DNA from the potato 1017 and tomato C31 parents, somatic hybrid SH 3117, BC1 6701 and BC2 6731-14 were digested with BglII (1) and EcoRV (2). The fragments were separated on a CHEF gel under conditions that allow resolution of fragments in a range up to 2 Mb. The fragments were blotted and subsequently hybridised with a probe containing the telomeric repeat from *Arabidopsis thaliana* (a) and a probe containing the TGR1 repeat (b).

Fate of the TR and TGR1 repeats in the somatic hybrid, the BC1 and the BC2 plants

Unexpectedly, a dramatic loss of TGR1 and tomato specific TR bands was observed in the somatic hybrid (Fig. 5a and 5b), which was previously shown to contain a full complement of tomato chromosomes by GISH and RFLP analysis (Jacobsen *et al.*, 1995). If there is no change of DNA sequences in the tomato chromosomes of the somatic hybrid during the cell fusion procedure, hybridisation patterns of TR and TGR1 identical to the tomato parent are to be expected for the somatic hybrid. However, the size of the bulk TR bands was found to drop from 50-250 kb down to 10-150 kb, while a number of high molecular weight bands with sizes more than 700 kb were absent in the somatic hybrid. Similarly, the number of the hybridisation bands of the TGR1 probe was dramatically reduced and the large fragments were not detected in the somatic hybrid. These results suggest that either chromosome fragments with large TR-TGR1 sequences are eliminated or the TR-TGR1 sequences are dramatically reduced in size.

In order to gain cytogenetic evidence that could support the findings by PFGE, a two-colour FISH with probes of digoxigenin labelled TGR1 and biotin-labelled TGR2 was performed on metaphase complements from the somatic hybrid, the BC1 and BC2 plants (Fig. 6a, 6b and 6c). The TGR2 probe (red colour signals) known to paint large parts of most tomato chromosomes (Ganal *et al.*, 1988) was used to distinguish the parental chromosomes. FISH revealed in the somatic hybrid 22 chromosomes containing the TGR2 probe, with 34 telomeric TGR1 sites in stead of the expected 40, based on nine pairs of chromo-

somes carrying TGR1 at both end and two pairs at one ends in original tomato chromosomes (Zhong *et al.*, 1998). At least ten chromosomes in the somatic hybrid appeared to have the TGR1 signals at only one end (see arrows in Fig. 6a), indicating that, indeed, 6 telomeric TGR1 sites were lost in this plants. Also in the BC1 plant (Fig. 6b), only 13 telomeric TGR1 signals were detected rather than the 16 sites expected (two at short arm of a pair of chromosome 1, eight at both arms of a pair of chromosome 3 and 6, six at both arms of a single chromosome 8, 9 and 10). Based on the TGR1 signals and the karyotype of tomato chromosomes, three TGR1 sites which were lost in the BC1 plant were at the telomeric sites of the long arm of a chromosome 6, 9 and 10. The results can easily explain why a TGR1 telomeric site was not present at the long arm of the tomato chromosome 6 in the BC2 plant (Fig. 6c).

Discussion

The combination of genomic *in situ* hybridisation and FISH with TGR1 as probe has clearly demonstrated the existence of a small potato segment in the alien chromosome of the monosomic addition plant #6731-14. Exchanges between tomato and potato chromosomes are no exception and have been mentioned for several intergeneric and interspecific hybrids since the introduction of genome painting. Translocation chromosomes were repeatedly described in the tomato (+) potato hybrids (Jacobsen *et al.*, 1995) and are likely the result of an exchange between tomato and potato chromosomes via breakage and fusion events during or shortly after protoplast fusion. A second explanation for a compound chromosome comes from meiotic studies of pairing configurations at pachytene and multivalent associations at metaphase I, demonstrating the formation of recombinant chromosomes through homoeologous recombination between tomato and potato chromosomes (de Jong *et al.*, 1993; de Jong *et al.*, 1995; Garriga-Calderé *et al.*, 1997, 1998). As the nature of the tomato chromosomes 6 in the somatic hybrid and the BC1 plant is still to be ascertained, no conclusive explanation for the origin of the potato segment in the tomato chromosome of the monosomic addition can be given.

GISH in the monosomic addition unveiled in the recombinant chromosome at pachytene a distal, 2 μm short potato segment, spanning 7% of its long arm. This segment apparently exceeds the boundary of the chromosome region covered by the 14 RFLP markers of the linkage map for that chromosome. It follows that the site of this breakpoint was positioned anywhere between the proximal border of TGR1 and the most distal RFLP marker (TG221). With the disappearance of the distal TGR1 site on the long arm, only two loci of this tomato satellite repeat remained for high resolution FISH analysis, *i.e.*, the distal TGR1 on its short arm (together with the TR) and a smaller, interstitial site on the long arm. Both

domains were shown to co-localise with chromomeres as observed at pachytene (Ramanna and Prakken 1967).

Our measurements of fluorescent tracks on extended DNA fibre revealed for the short arm TR an average length of 5.1 ± 1 µm, and that for its contiguous TGR1 of 125 ± 10 µm, values that, on the basis of a stretching degree of 3.27 kb/µm (Fransz *et al.*, 1996), correspond to 16 ± 3 kb and 408 ± 33 kb, respectively. The interstitial site amounts to 50 ± 3 µm, which equals 163 ± 10 kb. If we compare these values to the original bulk data for all extended DNA fibres from whole tomato nuclei as demonstrated in a previous report (Zhong *et al.*, 1998), the conclusions seems justified that the group IIC in that study, with average sizes for TR of 15.3 kb and for TGR1 of 404.5 kb represents the short arm TR/TGR1 of chromosome 6. Likewise, the interstitial TGR1 on the long arm should correspond to group IVb in that overview for all tomato TR/TGR1 combinations.

The results of the analysis of large fragment patterns as resolved by PFGE were in line with the size estimates of the TR and TGR1 repeats as far as the monosomic addition plant is concerned. The *Bgl*II fragments for both repeats in this BC2 plant were about 560 kb, indicating one restriction site at a 135 kb (= 560 - 425) distance from the proximal TGR1 border and no restriction site at all between both repeats. The *Eco*RV fragments were even smaller measuring 470 kb, indicating the closest restriction site on a distance of c. 45 kb from the TGR1 border. The minor bands of 580 and 600 kb may be interpreted as the result from flanking restriction sites on either site the interstitial long arm TGR1 segment. The molecular organisation of both repeat regions on tomato chromosome 6 with putative positions of the *Bgl*II and *Eco*RV restriction sites is schematically depicted in figure 7.

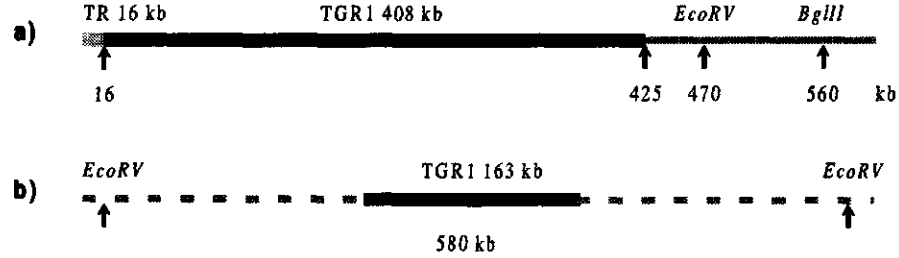

Figure 7. Schematic drawing of the molecular organisation of the TR and TGR1 sequences in the tomato chromosome 6 on basis of the data from Fibre-FISH and PFGE. a) Molecular organisation of the TR/TGR1 at the telomere region of the short arm. b) Molecular organisation of the TGR1 at the interstitial site of the long arm.

Rather surprising were the results of the Southern analysis of restriction digested high molecular weight DNA fragments of the parental species, their somatic hybrid and the BC1. There was a conspicuous difference for the TR profiles between tomato and potato, with generally far higher repeat sizes for the former species. In addition, only the tomato

lane showed TGR1 bands confirming the species specificity of this repeat. Zhong *et al.* (1998) calculated on the basis of extended DNA fibres signals, that the TR repeat in tomato varied from 15.5 to 216 kb, and that of TGR1 from 88 to 1330 kb. Remarkable were the differences in TR and TGR1 lengths in the somatic hybrid compared with the tomato parent, showing a band shift and/or complete loss had occurred for both repeats. The new hybridisation fragments that appear in the BC1 carrying six different tomato chromosomes suggest that the process modifying repeat lengths still proceeds in the BC1 generation. We assume that merging the genomes of tomato and potato protoplasts in a somatic hybrid triggers in some way large scale genome instability leading to a diminishing of at least two repeat families. The observation that FISH preparations with mitotic metaphase complements of the somatic hybrid reveals 34 TGR1 sites rather than the expected 40 further strengthen our assumption on loss and reduction of tandem repeats.

Comparable genomic changes in newly synthesised polyploid hybrids of *Brassica* were reported by Song *et al.* (1995), showing loss and/or gain of restriction fragments and appearance of novel fragments in F_2 to F_5 individuals. A very similar phenomenon was described for newly synthesised amphiploids with the genomic constitution of hexaploid wheat, revealing rapid, non-random elimination of specific, low-copy, probably non-coding sequences (Feldman *et al.*, 1997). An other example of genome changes at the molecular level was shown for rDNA tandem repeats in *Gossipium* hybrids (Wendel *et al.*, 1995). Such sudden modifications of possibly specific sequences were considered to be brought about by DNA methylation, whether or not resulting from ploidy changes (Scheid *et al.*, 1996).

The important question as to whether the TR and TGR1 repeats in the tomato chromosome 6 remain unaffected during the somatic hybridisation and back-crossings remains still to be answered. It is not clear yet whether the TR and TGR1 repeat lengths in the alien chromosome of the monosomic addition do actually correspond to the sites of the native tomato chromosome 6. The Southern analysis of high molecular weight DNAs suggests that especially the larger tomato TR/TGR1 repeats get lost in some way during the formation of the intergeneric hybrid, leaving the relatively small TR/TGR1 repeats in this chromosome unaffected. Consequently, one may speculate that the short arm distal TR/TGR1 combination, evidently one with the shortest TRs and an average TGR1 repeat size, has more chance to survive the general loss of repeats in the hybridisation process.

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Chapter 6

PHYSICAL MAPPING AND LOCALISATION OF BACTERIAL ARTIFICIAL CHROMOSOMES SPANNING THE NEMATODE RESISTANCE GENE *Mi* TO THE SHORT ARM OF TOMATO CHROMOSOME 6 BY FISH

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Abstract

Fluorescence *in situ* hybridisation (FISH) was used to construct a physical map of two BAC clones, BAC1 and BAC3, spanning the nematode resistance gene *Mi* in tomato and to assign the BAC clones to chromosomal locations. The BAC clones were selected from a BAC library constructed from a tomato YAC clone, YAC1256, which carries the *Mi* gene. The molecular sizes of the inserts of the BAC clones as determined by pulsed field gel electrophoresis, were 57 kb and 50 kb, respectively. A physical map showing the relative position of the two BACs was constructed by Fibre-FISH of digoxigenin labelled BAC1 and biotin labelled BAC3 to tomato genomic extended DNA fibres from young leaf nuclei. The green fluorescent signals of BAC1 were directly flanked by red signals of BAC3 with a overlap region of 12 kb. The two overlapping BAC clones were concluded to cover a 95 kb genomic region around the *Mi* gene. The BAC clones were further mapped to the euchromatic region close to the border of heterochromatin at the short arm of chromosome 6 by two-colour FISH to pachytene chromosome preparations. The results show that FISH is a fast tool for direct physical mapping and chromosome localisation of DNA markers related to interesting genes.

Key words: Fluorescence *in situ* hybridisation, FISH, Fibre-FISH, pachytene chromosomes, bacterial artificial chromosome, BAC, Nematode resistance gene, *Mi*, tomato

Introduction

Root-knot nematodes (*Meloidogyne spp*) cause very serious damage to many crops throughout the world. Conceivably, molecular cloning and tailoring of plant genes conferring resistance against nematodes have 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 in a combined effort of three laboratories (Kaloshian *et al.*, in press; Vos *et al.*, to be published). Thus, more than 50 years after the identification of host-encoded nematode resistance, the stage has been set for an in-depth analysis of the nematode-tomato interaction and the subsequent application of the cloned resistance gene in practical breeding.

In the early 1940s, 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* with the help of an

embryo culture (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 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* was dominant and segregated as a single major locus (Gilbert and McGuire, 1956), that was mapped on chromosome 6 in tight linkage (~1 cM) to 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-resistance in tomato breeding programs. Based on deletion mapping (Khush and Rick, 1968), *yv* could be assigned to the pericentromeric heterochromatin region of the long arm of chromosome 6 with 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 the *yv* locus, *Mi* has always been marked 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, Weide *et al.*, 1993). However, on the basis of physical data from deletion mapping experiments using molecular markers tightly linked to *Mi* (van Wordragen *et al.*, 1994), preliminary evidence was presented showing *Mi* to be located on the short arm rather than on the long arm. This has been further substantiated recently by means of a detailed molecular mapping of numerous irradiation-induced deletions, that involved both the *Mi* gene and the morphological 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 this chapter, we have completed the map integration by establishing the chromosomal, cytogenetic map position of *Mi* as revealed by FISH to pachytene chromosomes using two BAC clones as probes derived from the *Mi* region. It is shown that *Mi* is indeed located on the short arm of chromosome 6 at the telomere proximal border of pericentromeric heterochromatin and euchromatin.

Materials and methods

Tomato BAC clones

A BAC library was constructed by Williamson *et al.* (unpublished) from YAC1265 which spans the *Mi* gene (Kaloshian *et al.*, in press; Vos *et al.*, unpublished). Two BAC clones, BAC1 and BAC3 were selected from the BAC library for physical mapping and chromosomal localisation by FISH. The BAC DNAs were isolated by an alkaline lysate method (Woo *et al.*, 1994) and further purified by a GlassMAX DNA isolation reagent system (Life Technologies). The tomato DNA inserts were released from the BAC clones by

NotI digestion and their molecular sizes were estimated by pulsed field gel electrophoresis for 20 hours at an initial pulsed time of 1 second and a final pulsed time of 10 seconds at voltage of 6.0 V/cm.

Probe labelling

The purified BAC1 and BAC3 DNAs were labelled with digoxigenin-11-dUTP and biotin-16-dUTP, respectively, by standard nick translation according to instruction 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; Zhong *et al.*, 1996b).

FISH

Two-colour FISH of BAC clones 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 probe was 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 colour 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 commercial 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. 2b) were aligned based on positions of overlapping parts.

Results

Molecular characterisation of BAC1 and BAC3

Restriction mapping analysis of BAC1 and BAC3 had already shown (Williamson et al., unpublished) that both clones are partially overlapping and are located near the centromere proximal end of YAC 1256, which spans most of the 650 kb introgressed *L.*

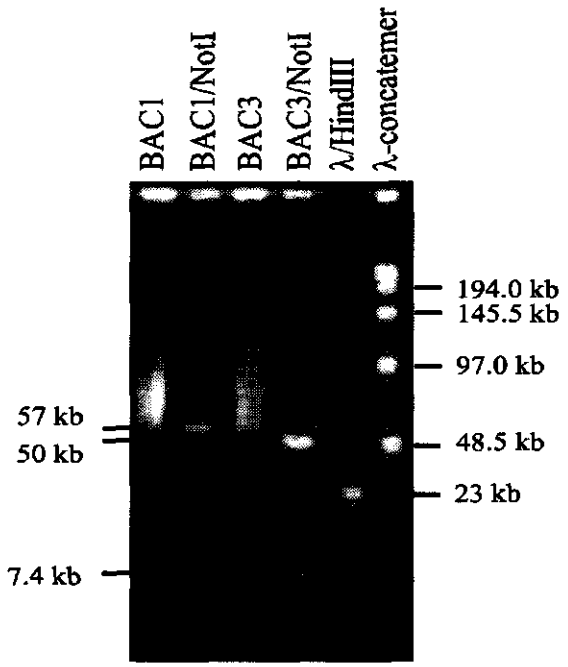


Figure 1. Pulsed field gel electrophoresis pattern of undigested and Not1 digested BAC1 and BAC3.

peruviannum chromosomal segment carrying *Mi* in cultivar Motelle (Vos *et al.*, to be published; see also Kaloshian *et al.*, in press).

The sizes of the *NotI* inserts of BAC1 and BAC3 were determined by PFGE and found to be 57 kb and 50 kb, respectively (Fig. 1). In order to establish the extent of overlap, the BAC's 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). 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. A microscopic photograph in an area of 100 × 80 mm^2 containing three Fibre-FISH signals is shown in Fig. 2a. On average, two to three Fibre-FISH signals were found per field of view using a 63 × microscope objective. 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 signal 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, Fig. 2b). Taking into account the stretching degree 3.27 kb/μm of the extended DNA fibres found previously (Fransz *et al.*, 1996), the molecular sizes of BAC1, BAC3 and the overlap were calculated 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 (Fig. 1). Accordingly, the two partially overlapping BAC clones were estimated to cover a 95 kb (57 + 50 - 12 = 95 kb) genomic region around the *Mi* gene (see schematic physical map in Fig. 2c).

Table 1. Fibre-FISH signal lengths and molecular sizes of the two BACs

BAC	Number of molecules	Length of the signals (μm)	Molecular size measured by FISH (kb)	Molecular size measured by 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	

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 found previously (Fransz *et al.*, 1996). Only very slight difference of the molecular sizes was found between the measurements by FISH to extended fibres and PFGE.

FISH mapping of BAC1 and BAC3 on pachytene chromosomes

To precisely localise the two BAC clones on the cytogenetic map of chromosome 6, a two-colour FISH experiment was performed on pachytene chromosome preparations using digoxigenin-dUTP labelled BAC1 and biotin-dUTP labelled BAC3, respectively. After washing at high stringency (0.1 × SSC at 60°C), the BAC's were found to be co-localised at the short arm of a chromosome that, on the basis of its morphology, was identified as chromosome 6. Hybridisation signals appeared in the euchromatic region very near the border with the pericentric heterochromatin (see Fig 3a, 3b and 3c) at a fractional length of the short arm of 0.4 (a fraction from the FISH signal to telomere over the total chromosome arm length). At late pachytene stage, this chromosome 6 has a length of 36.9 μm (Fig. 3c) with 2.3 μm and 23.6 μm corresponding to the euchromatin, and 4.3 μm and 6.7 μm to the heterochromatin at the short and long arm, respectively.

Figure 2. Physical mapping of BAC1 and BAC3 by Fibre-FISH. The green and red fluorescent tracks represent hybridisation signals of BAC1 and BAC3, respectively. Short stretches of yellow fluorescence indicated co-localisation of green and red signals and are interpreted as overlapping region of the two BACs. **a.** a microscopy picture with an area of 100 x 80 mm^2. **b.** ten hybridisation signals manually aligned based on position of overlap parts. **c.** schematic representation of the physical map of the two BACs. bar = 10 mm

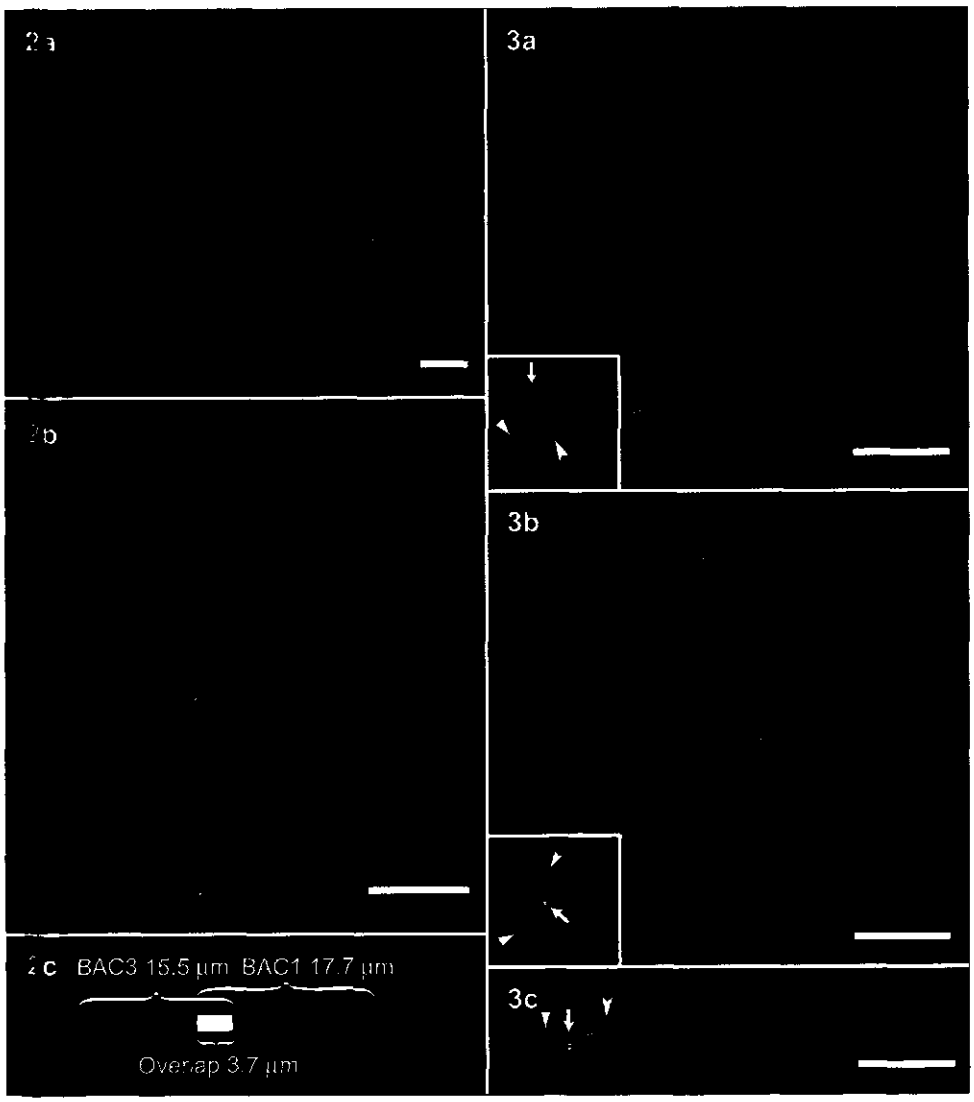
Figure 3. Localisation of the BAC1 and BAC3 related to the Mi gene by FISH to pachytene chromosomes. **a** and **b.** a pachytene complement with FISH signals at the short arm of chromosome 6. The green and red signals, representing BAC1 and BAC3, respectively, co-localised to the same position. **c.** a single pachytene chromosome 6 carrying hybridisation signals at the euchromatic region close to border of heterochromatic regions at the short arm. The chromosome length is 36.9 µm. bar = 10 µm. The arrows indicate the positions of hybridisation signals. The arrow heads and triangle point to the positions of centromere and telomere, respectively.

Discussion

In this paper we have provided direct physical evidence showing that the root-knot nematode resistance gene *Mi* is indeed located on the short arm of chromosome 6, as already indicated by molecular mapping (see Fig. 4B, Liharska *et al.*, 1997; also see van Wordragen *et al.*, 1994 and 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. 4A, Weide *et al.*, 1993).

With a genetic distance of only 1.1 cM between *Mi* and *Aps-1lyv*, the actual position of *Mi*, at least 30 Mb away from these loci (see below) at the other side of the centromere near the telomere proximal border to 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 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 visualised in a cytogenetic analysis in which the frequency and distribution of recombination nodules (RNs) was measured in synaptonemal complexes (SCs; Sherman and Stack, 1995). As RNs are associated with sites of crossing over, the appearance of RNs directly reflects the distribution of recombination events at different chromosomal regions. Thus, a high resolution RN map of the tomato genome has been constructed (Sherman and Stack, 1995). As shown in Fig. 4C, among the 400 SCs analysed, virtually no RNs were found in the heterochromatic region of the short arm and only a few RNs 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.


2a
2b
2c BAC3 15.5 µm BAC1 17.7 µm
Overlap 3.7 µm
3a
3b
3c

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 (also see Chapter 7). In considering a DNA content of approximately 950 Mb for tomato haploid genome (Arumuganathan and Earle, 1991) packaged into 12 chromosomes spanning a total length of 483 µm at late pachytene stage (Ramanna and Prakken, 1967), 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), accounting for 116 µm and 367 µm of the chromatin lengths (Ramanna and Prakken, 1967), respectively, the DNA compactness in heterochromatin corresponds to 6.3 Mb/µm and in euchromatin to 0.6 Mb/µm. Taking the microscopic length measurements for chromosome 6 (Fig. 3C) into consideration, the heterochromatin at the short arm accounts for at least 27 Mb of DNA, with *Mi* being located at the junction with euchromatin (Fig. 4D). Accordingly, the genetic distance of 1 cM between *Mi* and *Aps-1/yv* corresponds to a physical distance of at least 30 Mb, a base pair-to-cM relationship more than 40 fold higher than the average value of 750 kb/cM of the tomato genome (Tanksley *et al.*, 1992).

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 crucial before embarking on the positional cloning of a target gene merely on the basis of its very tight linkage to molecular markers.

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 identifying 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.

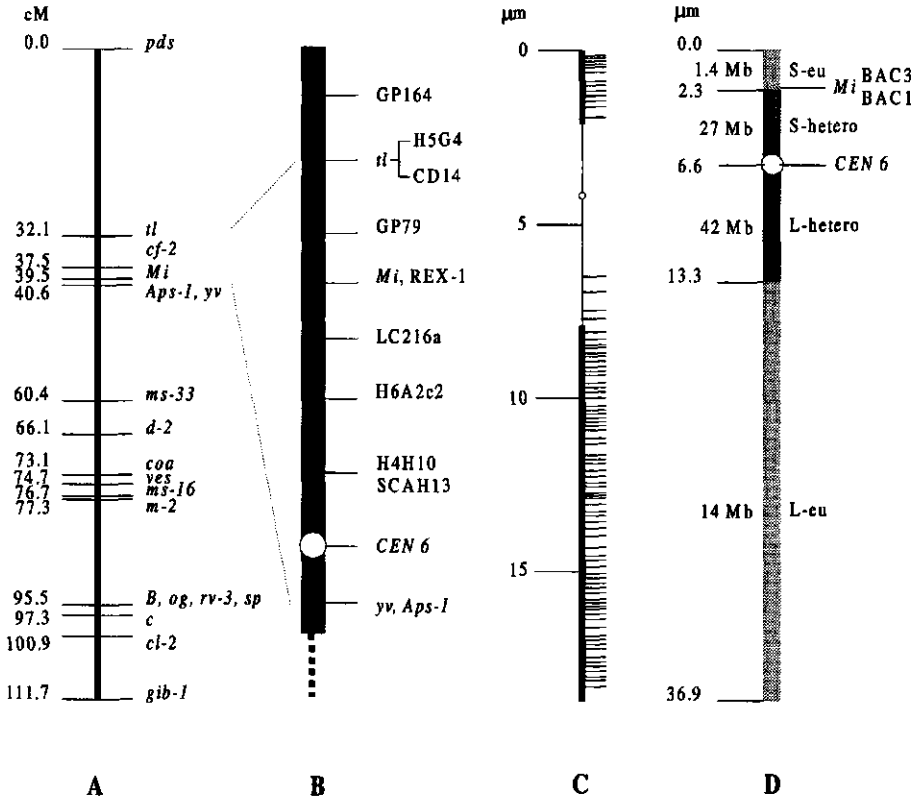


Figure 4. a. A classical genetic linkage map of tomato chromosome 6 showing genetic distances between classical markers (Weide *et al.*, 1993). **b.** A molecular map established by deletion mapping showing molecular marker order in the region around the centromere of tomato chromosome 6 (Liharska *et al.*, 1997). **c.** A recombination nodule map of tomato chromosome 6 showing the distribution of RN along the chromosome (Sherman and Stack, 1995). A thick line represents euchromatin and a thin line represents heterochromatin. The distance between each horizontal thin line equals one map unit measured from the centromere. **d.** A ideogram of the pachytene chromosome in Fig. 3c showing the lengths and DNA contents in the euchromatin and heterochromatin in both short arm and long arm. The *Mi* gene is placed on the border of the euchromatin and heterochromatin in the chromosome.

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Chapter 7

GENERAL DISCUSSION: CURRENT ACHIEVEMENTS IN TOMATO GENOME MAPPING BY FISH

Over the last century, tomato has been one of the most favourite species for plant genome mapping. The crop provides numerous morphological traits, has excellent chromosome morphology and now also displays a detailed genetic map comprising over a thousand molecular markers. However, studies on the integration of the genetic map with the chromosomal maps was initially hampered by limited accuracy of cytogenetic mapping tools such as deletion mapping and fluorescence *in situ* hybridisation (FISH). In the 1980s and early 1990s, genome mapping by FISH was mainly carried out on polytene chromosomes of *Drosophila* species (Rudkin and Stollar, 1977) or on species with large mitotic metaphase chromosomes, such as human and wheat, whether or not in combination with chromosome banding techniques. Along with small sized mitotic chromosomes found in *Arabidopsis thaliana*, the other model plant species for molecular genetics, those of tomato have for some time been regarded as unsuitable for FISH studies. However, during the period of this thesis, several technical advancements in FISH technology have been established in both our research group and that of other laboratories.

Highly repetitive DNA families occupying large segments in the chromosomes can be roughly mapped on mitotic metaphase chromosomes (Chapter 2, Xu and Earle, 1994; Zhong *et al.*, 1996a). Using the highly decondensed pachytene chromosomes as hybridisation targets, the different classes of repetitive sequences can more precisely be assigned to specific chromosome regions including centromeres, telomeres, heterochromatin and euchromatin (Chapter 2, 3 and 4; Zhong *et al.*, 1996a, 1996b and 1998; Xu and Earle, 1996a and 1996b). Application of FISH to pachytene chromosomes also allows the mapping of single copy DNA clones containing interesting genes to specific chromosomal locations and integration of the molecular genetic map into a chromosomal map (Chapter 6).

Studies of DNA sequence organisation at the molecular level can be directly performed by FISH to stretched individual DNA fibres, as shown for the molecular arrangement of the repeated unit of 18S and 25S ribosomal sequences along DNA molecules (Fransz *et al.*, 1996) and the positional relationship of two telomere specific repeats in individual telomere domains in the tomato genome (Chapter 4 and 5; Zhong *et al.*, 1998). Fibre-FISH also enables rapid construction of a physical map showing the relative position of isolated DNA clones in relation to orientation, overlaps and gaps in a single experiment (Chapter 6). These powerful FISH techniques together with conventional molecular biological techniques will result in high resolution physical maps of regions containing unique and repetitive DNA sequences.

The sensitivity and resolution of FISH mapping in tomato genome

In applying FISH to tomato, irrespective of the kind of DNA target studied, two major factors, detection sensitivity and mapping resolution, are crucial in determining the mapping effectiveness and accuracy, respectively. The detection sensitivity of FISH techniques is defined as the smallest DNA sequence on target which is detectable beyond doubt (Wiegant, 1994a). The consensus among cytogeneticists is that when at least 10-20% of observed targets display unequivocal hybridisation signals on identical positions of the two sister chromatids of a chromosome or on the homologous positions of the parental chromosomes, the FISH results are considered as beyond question. Many factors can influence the detection sensitivity, including the methods used for making microscopic preparations, labelling and hybridising probes to targets, detecting signals by immuno-fluorescent reagents, as well as the physical properties of the fluorescence microscope. If the whole procedure is carried out under optimal conditions, FISH sensitivity remains dependent mainly on the degree of DNA condensation as less condensed DNA structures are supposed to allow higher accessibility of the targets. Such a parameter thus reflects the DNA content per unit length of the hybridisation targets.

Under the conventional fluorescence microscope, the sensitivity of standard FISH to human metaphase chromosomes is in a range of cosmid-sized targets of about 30 kb (Lichter *et al.*, 1990; Wiegant *et al.*, 1991 and 1993), although smaller sizes of 1 to 3 kb can be detected with a cooled, integrating slow-scan CCD camera (Wiegant *et al.*, 1993). Experimental evidence shows that application of FISH to tomato pachytene chromosomes can detect at least single copy sequences as small as 50 kb, as shown for the mapping of a BAC clone at the junction region of euchromatin and heterochromatin in the short arm of chromosome 6 (Chapter 6). There are large differences in DNA condensation between the heterochromatin and euchromatin regions at the pachytene stage, and obviously these regions have different detection sensitivities.

When FISH was applied to extended DNA fibres from tomato nuclei, a plasmid clone containing a 700-bp sequence from the region of the 25S ribosomal gene appeared as single fluorescent dots (Fransz *et al.*, 1996), implying that Fibre-FISH can detect target sequences as small as seven hundred basepairs (bp). A novel FISH technology, known as molecular combing technique (Bensimon *et al.*, 1994), was recently developed for stretching cloned DNA molecules. We adapted this method in our laboratory to spread DNA fibres from YAC, BAC and phage-λ clones (unpublished data).

In summary, with the decrease of DNA condensation on various targets, the detection sensitivity of the FISH techniques in tomato increases from several hundreds of kbp on metaphase chromosomes, down to tens of kbp on pachytene chromosome and several hundreds of bp on extended DNA fibres and stretched DNA clones (see Table 1).

Table 1. Mapping resolution, detection sensitivity and applications of FISH to various DNA targets in tomato

Cytogenetic data of the tomato genome	mitotic metaphase chromosome		Pachytene chromosome		Extended DNA fibre	Isolated DNA clone
	heterochromatin	euchromatin	heterochromatin	euchromatin		
Haploid genome size [a]	950 Mb		950 Mb		950 Mb	
Fraction of DNA in heterochromatin/euchromatin [b]	77%	23%	77%	23%		
DNA content in hetero-/euchromatin	732 Mb	218 Mb	732 Mb	218 Mb		
Length of hetero-/euchromatin [c]	26 µm	9 µm	116 µm	368 µm		
DNA condensation [d]	28 Mb/µm	24 Mb/µm	6.3 Mb/µm	0.6 Mb/µm	3.27 kb /µm [e]	2.3 kb/µm [e]
Resolution of the light microscopy	0.2 µm		0.2 µm		0.2 µm	0.2 µm
FISH mapping resolution	4-5 Mb [e]	4-5 Mb [e]	1.2 Mb [e]	120 kb [e]	~ 1 kb [e]	< 1 kb [e]
Detection sensitivity	> 100 kb	>100 kb		< 50 kb [e]	< 0.7 kb [e]	< 0.5 kb [e]
Applications of FISH to different targets	• comparative analysis of genome structure with other species by GISH; • rough localisation of repetitive sequences on chromosomes		• assignment of repetitive and single copy sequence to special chromosome regions; • studies of high order organisation in different chromosome regions		• fast construction of physical maps; • analysis of molecular organisation in a genomic region up to 2 Mb	• fast construction of restriction maps; • analysis of molecular organisation of a defined region in details

a)Arumuganathan and Earle, (1991); b) Peterson *et al.*, 1996; c) Ramanna and Prakken (1967); d) the average DNA condensation is deduced from the DNA content divided by the length of the corresponding chromosome segment; e) Fransz *et al.*, (1996) and Weier *et al.*, (1995).

Similar to the detection sensitivity, mapping resolution is also determined by the degree of DNA condensation on the targets at a given FISH condition. By definition, this parameter is defined as the smallest physical distance between two different target sequences that can be spatially resolved by the microscope used in FISH studies (Wiegant *et al.*, 1994a). At a maximum spatial resolution of a conventional fluorescence microscope of 0.2 μm, a series of human molecular cytogenetical experiments established the mapping resolution by FISH on various hybridisation targets. A distance of 1-3 Mbp could be resolved on metaphase chromosomes (Lichter *et al.*, 1990). When mechanical force was used to stretch metaphase chromosomes to 5~20 times their natural lengths, a separation of 200 kb could be determined on these chromosomes (Haaf and Ward, 1994). A higher resolution of 50-100 kbp was reported on less condensed chromatin in interphase nuclei (Trask *et al.*, 1989). With fibre-FISH, the mapping resolution amounts to about 1 kb (Wiegant *et al.*, 1992; Parra and Windle, 1993; Haaf and Ward, 1994), which closely correspond to the theoretical resolution of 2.94 kb/μm in the Watson-Crick DNA double helix model.

So far, no systematic survey as to the level of attainable FISH resolution in tomato has been conducted. However, a theoretical estimate (see Table 1) can be made on the basis of literature data about DNA condensation of tomato chromosomes. After establishing the DNA contents in different chromatin regions (Peterson *et al.*, 1996) and their related chromatin lengths (Ramanna and Prakken, 1967), the DNA condensation has been calculated for heterochromatin and euchromatin regions of both metaphase and pachytene chromosomes. In comparison to a slight difference of DNA condensation of 28 Mb/μm and 24 Mb/μm between heterochromatin and euchromatin on metaphase chromosomes, a dramatic variation of the DNA condensation of 6.3 Mb/μm in heterochromatin and 0.6 Mb/μm in euchromatin is found for pachytene chromosomes. At a resolution of 0.2 μm of a fluorescence microscope, the mapping resolution ranges from 4-5 Mb for mitotic metaphase chromosomes, whereas such values for heterochromatin and euchromatin in pachytene chromosomes varies from 1.2 Mb to 120 kb (Table 1).

Dissection of the tomato genome by FISH

As the levels of detection, sensitivity and mapping resolution increased using FISH techniques for various hybridisation targets, they created potential for a detailed analysis of chromosomal structure and genome organisation. Initially, this was applied in particular to the analysis of repeated sequences.

Ribosomal genes

The ribosomal gene cluster was among the first regions of the tomato genome to be studied by high resolution FISH mapping strategies. Upon isolation and molecular characterisation of the 45S and 5S ribosomal genes (Kiss *et al.*, 1988; Lapitan *et al.*, 1991), the 5S rDNA genes could be localised to the heterochromatic knob in the centromere region of chromosome 1 using FISH to pachytene chromosomes (Xu and Earle, 1996a). The major 45S rDNA site was located in the Nuclear Organiser Region (NOR) and flanking satellite and short arm regions of chromosome 2 (Chapter 2, Xu and Earle, 1996b). Minor sites of the 45S rDNA repeat classes were found in heterochromatin knobs of several chromosomes in some tomato cultivars (Xu and Earle 1996b).

Our studies with fibre-FISH revealed a direct microscopical view of the molecular organisation of the rDNAs. Using a probe containing the 5S rDNA sequences, a consistent pattern of continuous fluorescent strings of about 200 µm was found which corresponds to a molecular size of about 660 kb (Fransz *et al.* 1996), thus confirming the previous finding of the 5S rDNA sequence size in the tomato genome (Lapitan *et al.*, 1991). The tandemly repeated organisation of the 45S rDNA sequences was demonstrated in similar fibre-FISH experiments and showed a consistent pattern of alternating fluorescent signals with different probes for the 18S and 25S sequences (Fransz *et al.*, 1996).

Telomere repeats

A second region dissected in detail is described in Chapter 4 (also see Zhong *et al.*, 1998) and involves the organisation of individual telomere domains of the different tomato chromosomes. The two repeats of the distal chromosome regions, *viz.* the telomeric repeat TR and the subtelomeric repeat TGR1, were for the first time studied by fibre-FISH. Four different classes of the relationship between these two repeats, comprising 27 different TR/TGR1 combinations, have been resolved at the DNA level. Their chromosomal distribution was further analysed by FISH to pachytene chromosomes, revealing specific information of these repeats for each individual telomere domain. The results further show how the chromatin loops containing the distal DNA repeats are folded at their chromosome ends.

To investigate the telomere organisation on chromosome 6, a monosomic addition containing tomato chromosome 6 in a tetraploid potato background was used as study material. Through the compound nature of the alien chromosome, with a small potato segment attached to the long arm of the tomato chromosome, only two sites of the TGR1 repeat remain detectable: one at the distal end of the short arm and the other on the interstitial chromomere of the long arm. The origin of the short potato chromatin at the distal end of the long arm is unknown: it can originate from either homoeologous recombination in the meiotic prophase I of the somatic hybrid or its BC1, or from a translocation with any of the

potato chromosome. Again, the combination of FISH to pachytene chromosomes and extended DNA fibres and molecular size estimates by Pulse Field Gel Electrophoresis displayed a very detailed molecular organisation of TR and TGR1 (Chapter 5). The results present a typical example of how individual domains of repetitive DNA sequences in specific chromosomal regions can be analysed by a combination of FISH on various targets with standard molecular techniques and using the benefits of a monosomic addition.

Other repeat families

Although a functional centromere DNA sequence is still not available for use as a probe in FISH mapping, the first step towards the isolation of tomato centromeric sequences has been accomplished by mapping the centromeres of chromosomes 6, 7 and 9 (Van Wordragen *et al.*, 1994 and 1996; Frary *et al.*, 1996; Liharska *et al.*, 1997). Furthermore, radiation-induced deletion mapping (Weide et al., submitted) enabled the isolation of pericentromeric sequences of tomato chromosome 6 (CEN6). Remarkably, some of the sequences showed homology to the human centromeric satellite III sequences and to the CENP-B binding box from mammalian satellites, suggesting that these sequences may be part of the components of tomato functional centromeres or pericentromeric heterochromatin. Once DNA sequences of core elements of tomato centromeric and pericentromeric sequences are isolated, molecular and chromosomal organisation of the tomato centromeres can be analysed in detail by FISH to pachytene chromosomes and extended DNA fibres.

In addition to the ribosomal genes, telomeres and centromeres, another important feature of chromosomes is the structure and organisation of heterochromatin and euchromatin. By definition, euchromatin becomes decondensed at the metabolically active stages of cell division, *viz.*, telophase, interphase and prophase, whereas heterochromatin remains condensed throughout the whole cell division cycle (Heitz, 1929). Hence, heterochromatin and euchromatin cannot be discerned at metaphase, whereas at pachytene and mitotic prophase chromosomes exhibit highly morphological differences between darkly stained heterochromatin and faintly stained euchromatin. This differentiation pattern is most conspicuous in tomato and can directly be seen in unstained preparations under the phase contrast microscope, or under bright field illumination upon staining with aceto carmine, Giemsa and other classical staining methods. A special class of heterochromatin, the so-called constitutive heterochromatin (Brown, 1966) can be revealed with the BSG- or C-banding technique. As to tomato, small specific C-band areas occur in the proximal heterochromatin regions, and the distal knobs of the chromosomes 1-6, and 8, 9 and 11 (L.P. Pijnacker and M.S. Ramanna, pers. commun.).

Incubation of chromosomes with DAPI or other fluorescent dyes exhibits heterochromatic areas as prominent fluorescent blocks, while euchromatin shows only faint fluorescence. Once pachytene chromosomes became suitable for FISH (Zhong *et al.*, 1996a and

1996b; Xu and Earle, 1996a and 1996b), it was possibility to distinguish the characteristics of DNA composition and chromosomal organisation between heterochromatin and euchromatin. For example, FISH to pachytene chromosomes showed that THG2, a member of the *HindIII* repeat complex, is a heterochromatin specific dispersed repeat (Zhong *et al.*, 1996a).

Table 2. Estimation of DNA content in heterochromatin and euchromatin of pachytene chromosomes in tomato and *Arabidopsis thaliana*

Species	Tomato	*Arabidopsis*
Genome size	950 Mb [a]	110 Mb
Average DNA content of a chromosome	950/12 = 79 Mb	110/5 = 22 Mb
Average chromosome length	483 [b]/12 = 40 μm	331 [d]/5 = 66 μm
Average heterochromatin length	115.7 [b]/12 = 9.6 μm	23.4 [d]/5 = 4.7 μm
Average euchromatin length	367.6 [b]/12 = 30.6 μm	307.8 [d]/5 = 61.6 μm
DNA condensation in heterochromatin	60.8/9.6 = 6.3 Mb/μm	0.7 [e] Mb/μm
DNA condensation in euchromatin	18.1/30.6 = 0.6 Mb/μm	0.3 [e] Mb/μm
DNA content in heterochromatin[f].	79 × 77% [c] = 60.8 Mb	4.7 × 0.7 = 3.3 Mb
DNA content in euchromatin[f]	79 × 23% [c] = 18.1 Mb	61.6 ×0.3 = 18.4 Mb

a. haploid tomato genome size (Arumuganathan and Earle, 1991)
b. total length of pachytene complements, heterochromatin and euchromatin in the tomato genome (Ramanna and Prakken, 1967)
c. fraction of DNA content in heterochromatin and euchromatin (Peterson *et al.*, 1996)
d. total length of pachytene complements, heterochromatin and euchromatin in the *Arabidopsis* genome (Fransz *et al*, 1998)
e. DNA condensation in heterochromatin and euchromatin deduced on basis of FISH mapping of a YAC contig of 2.7 Mb in the regions of the short arm of chromosome 4 in *Arabidopsis* (Fransz *et al.*, 1997).
f. DNA contents per chromosome

Recently, methods for preparing pachytene chromosomes have become available in *Arabidopsis thaliana* (Ross *et al.*, 1996). Combined with FISH using chromosome specific probes, a detailed pachytene karyotype of *A. thaliana* was presented, showing the lengths of chromosome arms, satellites, heterochromatin, euchromatin, and chromomeres for each chromosome (Fransz *et al.*, 1998). A comparison of the pachytene karyotype of *Arabidopsis* and tomato (see Table 2), elucidated several interesting relations. (1) The total cell complement measures 331 μm and 483 μm for *Arabidopsis* and tomato, respectively, length values that are disproportional to their respective genome sizes, amounting 110 Mb for *Arabidopsis* and 950 Mb for tomato. It follows that overall DNA condensation in tomato chromosomes is much higher than that in *Arabidopsis*. (2) In tomato, DNA in heterochromatin is ten fold more condensed compared to that in euchromatin, while in *Arabidopsis* they differ only by a factor of seven. (3) The major difference in DNA content per chromosome is due to the relative contribution of heterochromatin per chromosome, which is 60.8

Mbp in tomato and 3.3 Mb in *Arabidopsis*. These findings support a general conclusion that DNA abundance in plant genomes should mainly be attributed to repetitive DNA sequences located in the heterochromatin regions (Dean and Schmidt, 1995). FISH techniques have thus demonstrated to be an invaluable tool for molecular analysis of DNA organisation at the chromosomal level with respect to heterochromatin and euchromatin.

A full bar-code map for each tomato chromosome: a future perspective

After the tomato genome has been subdivided into minute, subchromosomal regions, each with their own domains of single copy sequences or repeat DNA families, integration of each part will give an overall picture of the chromosomal organisation at the molecular level. These so called "bar-code" maps which are already described for human chromosomes (Lengauer *et al.*, 1993; Florijn *et al.*, 1995; Müller *et al.* 1997) will directly display the positions of major repetitive DNA sequences (including the telomeric repeat, subtelomeric repeats, centromeric repeats, satellite repeats, micro- and mini-satellite repeats, (retro-)transposons, and so forth) and their relation to its molecular, chromosomal and recombination maps. Information will be obtained, not only on the distribution of each repeat element on the chromosomes, but also about the molecular size and sequence organisation of individual domains. A detailed overview of the order of repeat sequences will be given in the map from one telomere, via its adjacent distal euchromatin, proximal heterochromatin, centromere, to the other telomere. Genes will then be precisely mapped on specific chromosomal regions and it seems possible to correlate gene organisation and repetitive DNA regions with chromatin structure. To build up such "bar-code" maps of chromosomes, FISH techniques on various DNA targets are indispensable tools. Monosomic addition of tomato chromosomes in a potato background are most useful towards this goal, especially to facilitate mapping of dispersed repeat families on individual chromosomes. In the near future, such a comprehensive chromosomal map will help us to better understanding the tomato genome organisation at the molecular level.

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Samenvatting

De afgelopen jaren is het onderzoek naar de moleculaire en genetische organisatie van plantenchromosomen in een enorme stroomversnelling geraakt. Waren zo'n tien jaar geleden de chromosomale posities van slechts een aantal genen op chromosomen bekend, tegenwoordig zijn van een groot aantal gewassen zeer gedetailleerde koppelingskaarten beschikbaar. Dergelijke kaarten geven de relatieve posities van honderden tot duizenden coderende en niet-coderende DNA sequenties met hun onderlinge genetische afstand aan. Naast deze genetische kaarten bestaan ook moleculaire kaarten waarin de fysische posities van alle genen en niet-coderende sequenties met hun precieze DNA-code staan weergeven. Van de zandraket (*Arabidopsis thaliana*), het paradepaardje onder de planten voor moleculair biologen en genetici is inmiddels de volledige basenvolgorde van een van de vijf chromosomen bekend.

Hoewel deze informatie over de organisatie van het genoom reeds tot een aantal nieuwe inzichten heeft geleid en dagelijks een stimulans vormt voor verder onderzoek aan plantengenen, ontbreekt er nog één element aan het totaalbeeld van een plantenchromosoom, namelijk de integratie van de "lineaire" informatie van de fysische en genetische koppelingskaart met de cytologische kaart, d.w.z. met een kaart die de hogere orde organisatie van het chromosoom laat zien. Een soort drie-dimensionale chromosoomatlas dus, waarin de informatie over posities van DNA sequenties staan aangegeven ten opzichte van chromosoomuiteinde en centromeer, en andere chromosoommarkers, zoals de sterk gecondenseerde chromomeren en heterochromatische gebieden, die gekenmerkt worden door onder meer grote aantallen repetitieve sequenties en moeilijker toegankelijk zijn voor gentranscriptie. Belangrijke vragen dienen zich aan: welke genen en niet-coderende sequenties bevinden zich dan in het heterochromatine en welke in het euchromatine? En hoe liggen de genen verspreid ten opzichte van niet-coderende gebieden? En hoe is deze informatie ruimtelijk georganiseerd in de kern?

Een eerste stap op weg naar deze integratie kan gezet worden met een techniek die het mogelijk maakt om direct de ligging van DNA sequenties op het chromosoom onder het microscoop zichtbaar maken. Deze techniek, fluorescentie *in situ* hybridisatie (FISH), wordt reeds enige tijd met succes toegepast bij dierlijke en humane organismen, maar staat bij het plantenonderzoek nog in de kinderschoenen. In dit proefschrift wordt een aanzet gegeven tot de toepassing van deze techniek in het genoomonderzoek van de tomaat (*Lycopersicon esculentum*). De FISH-techniek wordt toegepast op zowel mitotische chromosomen van worteltopmeristeem-preparaten als meiotische profase chromosomen (pachyteenchromosomen), afkomstig uit gespreide kernen van pollenmoedercellen afkomstig uit an-

theren. De FISH op pachyteenchromosomen blijkt een belangrijke verbetering in detectie, en resolutie van naast elkaar gelegen fluorescentiesignalen. Bovendien hebben pachyteenkernen als voordeel dat alle chromosomen kunnen worden geïdentificeerd op basis van specifieke heterochromatinepatronen. Naast deze pachyteen-FISH tehniek wordt nog een andere speciale techniek beschreven, de DNA-fibre FISH, waarmee als het ware via een vergrootglas een stafkaart van een bepaald chromosoomgebied met moleculaire detaillering zichtbaar wordt gemaakt.

Hoofdstuk 1 allereerst behandelt een overzicht van de voortgang die geboekt is in het genoomonderzoek van tomaat, zowel moleculair-genetisch als cytologisch. Dit onderzoek heeft een gezonde voedingsbodem gelegd waarop het FISH-onderzoek kon voortbouwen. De ontwikkeling van de FISH techniek wordt beschreven en de mogelijkheden geschetst die deze techniek biedt om dieper inzicht te krijgen in de organisatie van genetisch materiaal.

Hoofdstuk 2 beschrijft de ontwikkeling van een aantal protocollen voor de bereiding van chromosoompreparaten uit mitotisch en meiotisch celmateriaal ten behoeve van FISH-onderzoek in tomaat. Toepassing van deze techniek wordt toegelicht aan de hand van de kartering van een aantal verschillende repetitieve DNA elementen.

In hoofdstuk 3 worden vervolgens "stap-voor-stap" recepten beschreven voor de bereiding van chromosoompreparaten en "extended DNA-fibres" ten behoeve van de fysische kartering van DNA sequenties. Aangetoond wordt dat met deze FISH technieken gelijktijdig de posities van meerdere DNA elementen (telomeer repeat TR en subtelomeer repeat TGR1) zichtbaar gemaakt kunnen worden.

Hoofdstuk 4 gaat in detail in op de moleculaire en chromosomale organisatie van individuele DNA repeat domeinen, waarin al dan niet TR en TGR1 in voorkomen. Aangetoond wordt dat ieder van de twaalf tomaatchromosomen een eigen, specifieke organisatie van deze repeats kent. Zo worden bij de uiteinden van sommige chromosomen TR- en TGR1-tandem repeats gescheiden door een "spacer", terwijl op andere chromosomen deze repeats direct aan elkaar gekoppeld zijn. Bovendien komen er ook chromosoomuiteinden voor met alleen een TR domein en hebben drie chromosomen halverwege hun lange arm een TGR1-domein, zonder TR. Nauwkeurige analyse van de FISH signalen op de uiteinden van pachyteenchromosomen toont aan dat de TR repeat, die zich op het DNA molecuul aan het uiterste einde bevindt, naar binnen gevouwen ligt, waardoor de subterminaal gelegen TGR sequenties het uiteinde van het chromosoom afdekt.

In hoofdstuk 5 wordt beschreven hoe de moleculaire organisatie van repeterende DNA sequenties op een bepaald chromosom van tomaat zeer effectief bestudeerd kan worden door gebruik te maken van een aardappel (+) tomaathybride die naast zijn normale set van 48 aardappelchromosomen één extra chromosoom van tomaat bevat. Dit soort zoge-

naamde monosome addities zijn ontwikkeld door de vakgroep Plantenveredeling en vormen uniek materiaal voor het beantwoorden van specifieke vragen uit het genoom-onderzoek. In dit proefschrift is gebruik gemaakt van een monosome additie die het tomatenchromosoom 6 bevat. Multicolour FISH analyses van het soortvreemde chromosoom bracht aan het licht dat het uiteinde van de lange arm een kort stuk aardappelchromosoom had gekregen, dat door breuk of meiotische recombinatie met de aardappelhomoeoloog was ontstaan. Met behulp van zowel standaard moleculaire technieken (pulsed field gel electroforesis) en FISH op pachyteenchromosomen en extended DNA fibres kon de chromosoom 6-specifieke moleculaire en cytogenetische organisatie van de TR en TGR1 repeats vastgesteld worden. Bovendien kon worden aangetoond dat in de somatische aardappel (+) tomaathybride en daarvan afgeleide terugkruisingsplanten grote delen van de TR en TGR1 repeats verloren zijn gegaan.

Hoofdstuk 6 geeft een goed voorbeeld hoe de combinatie van FISH aan pachyteen en extended DNA fibres een krachtige nieuwe technologie is geworden in de genoomanalyse van tomaat. Het laat zien hoe de FISH techniek geschikt is voor het bepalen van de chromosomale posities van coderende sequenties. Hiertoe is als voorbeeld de positie van het resistentiegen tegen het wortelknobbelaaltje (*Mi*) van tomaat in kaart gebracht, daarbij gebruik makend van BAC klonen van dit gen. Het *Mi* gen bleek gelegen te zijn in het grensgebied van heterochromatine en euchromatine, halverwege de korte arm. De positie van *Mi* is verrassend gezien de enorme fysieke afstand tot het *Asp-1* gen op de lange arm waar het genetisch zeer nauw (± 1 cM) aan gekoppeld is. Berekeningen laten zien dat de fysieke afstand overeenkomt met een genetische afstand van minstens 30 Mb. Dat betekent een bp/cM relatie die 40x hoger is dan de gemiddelde verhouding (750 kb/cM) tussen deze twee afstanden voor het genoom van tomaat.

Hoofdstuk 7, tenslotte, bediscussieert de potentiële mogelijkheden die de verschillende FISH technieken bieden voor het genoomonderzoek van tomaat en andere planten. Er wordt daarbij onder meer aandacht besteed aan de gevoeligheidswinst en het hogere oplossend vermogen van deze technieken en een toekomstbeeld geschetst en waarin een volledige "streepjescode" voor elk individueel chromosoom van tomaat ontwikkeld wordt. Een dergelijke streepjescode toont de onderlinge ligging van de verschillende typen DNA sequenties langs het chromosoom zoals zich die onder de microscoop manifesteren.

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List of publications

Fransz P, Alonso Blanco C, Liharska T, Zhong XB, Peters A, Zabel P and de Jong JH. 1995. High resolution physical mapping in tomato and *Arabidopsis* by fluorescence *in situ* hybridization on extended DNA fibres. 12th International Chromosome Conference, Madrid, Spain, September 11-15, 1995. *Chromosome Research* 3 (Suppl. 1): 48-49.

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Curriculum Vitae

I was born in Chengdo, Sichuan Province, People's Republic of China on September 9 1963. I enjoyed my primary, secondary and high school from 1970 to 1980 in Deyan city, Sichuan Province, China. From 1980 to 1984, I studied in the Department of Biology, Peking University in Beijing, China and obtained my Bachelor degree in plant physiology and biochemistry. From 1984 to 1991, I worked as a research fellow in the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences in Beijing. From April 1991 to March 1993, I took the MSc course of Biotechnology in the Wageningen Agricultural University (WAU), the Netherlands and obtained my Master Science degree in the Department of Molecular Biology, WAU. My MSc thesis was supervised by Dr. Pim Zabel and involved the search of unique genomic fragments corresponding to a cDNA and a RAPD marker associated with the nematode resistance gene (*Mi*) in tomato. In June 1993, I started my Ph.D studies on "Tomato genome mapping by fluorescence *in situ* hybridisation (FISH)" in the Department of Molecular Biology, WAU, having Prof. Ab van Kammen as my promoter, and Dr. Pim Zabel and Dr. Hans de Jong as my co-promoters. In January 1998, I started my postdoctoral work on human genome analysis in Prof. David Ward's Laboratory in the Department of Human Genetics of Yale University Medical School, New Haven, USA.