

Chapter 2

Molecular Cytogenetics

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Abstract Cytogenetics has played a key role in the history of scientific research in the Brassicaceae since the start of the last century. The discovery of the *Brassica* “U’s Triangle” species, elucidation of phylogenetic relationships and investigations of chromosome evolution all contributed to building up the basic genomic understanding of the Brassicaceae we have today. The advent of molecular cytogenetics in this family in the last 20 years has led to a progressively greater understanding of the factors underlying chromosome dynamics and organisation, meiotic and mitotic mechanisms and cell division processes. In addition, linking molecular cytogenetics with other molecular techniques, such as marker studies, DNA sequencing and protein expression analysis, has bridged the gap between chromosomes and linkage groups, resulting in a wealth of new information in this family. Future prospects for molecular cytogenetics in the Brassicaceae are bright. The recent and imminent release of additional Brassicaceae genomes will greatly facilitate development of probes for fluorescent in situ hybridisation as well as a comprehensive understanding of gene expression and protein interactions during cell division.

Keywords Cytogenetics • Fluorescent in situ hybridisation • Chromosomes • *Brassica* • *Arabidopsis*

2.1 Introduction

Cytogenetics, literally “cell genetics”, traditionally refers to the study of chromosomes. Cytogenetics conventionally encompasses studies of chromosome number, structure and organisation, chromosomal aberrations and chromosome behaviour

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during mitosis and meiosis. Early cytogenetics studies were crucial in defining species and genera in the Brassicaceae, and for elucidating complex species relationships in the *Brassica* crop species and wild allies (Morinaga 1934; Nagaharu 1935; Mizushima 1980). However, the advent of molecular cytogenetics has opened up whole new avenues of exploration in this family. Molecular cytogenetics describes techniques made possible by breakthroughs in molecular genetics over the last 30 years or so, starting with radioactive labelling and genomic in situ hybridisation of DNA to chromosome spreads, and moving through to chromosome and protein labelling using fluorescently tagged antibodies and other molecules (Speicher and Carter 2005). Modern molecular cytogenetics offers a suite of tools suitable for integrating physical and linkage maps, observing protein expression and co-localisation during mitosis and meiosis, investigating chromosomal architecture such as nucleolar organisational regions (NORs), centromere and telomeres, identifying locations of genes and repetitive elements and molecular karyotyping in hybrid and mutant studies.

A great deal of molecular cytogenetic functionality is currently in use or in the process of development in the Brassicaceae. The Brassicaceae is informally known as the crucifer or cabbage family, and comprises approximately 3,700 species in about 338 genera (Warwick and Al-Shehbaz 2006). The bulk of this review will focus on the well-studied and agriculturally important *Brassica* genus and on model plant species *Arabidopsis thaliana*, as these comprise the species upon which the majority of molecular cytogenetics studies in the Brassicaceae have been pioneered. Molecular cytogenetics has been used extensively in the Brassicaceae to characterise and track chromosome behaviour in interspecific hybrids, synthetic canola lines, chromosome addition and genomic introgression lines and in experimental genotypes. Outcomes of molecular cytogenetics in this family include elucidation of nucleolar organisational regions (NORs) and characterization of other meiotic mechanisms, tracking and identifying genomic introgressions between *Brassica* species for breeding purposes and the integration of physical and genetic linkage maps.

The advent of next generation and third generation sequencing technologies facilitating the cheap and rapid sequencing of many *Brassica* and wider Brassicaceae species genomes looks to bring forth a new spectrum of uses for molecular cytogenetics in this family. Future prospects for molecular cytogenetics in the Brassicaceae will be discussed.

2.2 Classical Cytogenetics in the Brassicaceae

Classical cytogenetics, or the use of light microscopy to interrogate chromosome number, meiotic chromosome behaviour in natural species, haploids and interspecific hybrids, has taught us a lot about the Brassicaceae family. Classical cytogenetics provides a base upon which molecular techniques can build and expand on, and is still informative and relevant today in many poorly-examined clades. The unfortunately small size and relative lack of differentiation of most Brassicaceae

chromosomes relative to some other plant genera has provided somewhat of a handicap, particularly in the use of karyotyping through “C-banding”, or observation of heterochromatin patterns in chromosomes, but nevertheless many highly informative studies have been carried out using only classical cytogenetics. The most widely known use of cytogenetics in the Brassicaceae is the discovery of the relationship between the six agriculturally significant species *Brassica rapa* (buck choy, turnip), *B. nigra* (black mustard), *B. oleracea* (cabbage, cauliflower, broccoli), *B. juncea* (Indian mustard), *B. napus* (rapeseed, canola) and *B. carinata* (Ethiopian mustard). Although this relationship was first elucidated almost incidentally by T. Morinaga in a paper entitled “Interspecific hybridization in *Brassica* VI. The cytology of F_1 hybrids of *B. juncea* and *B. nigra*” in table form (Morinaga 1934), it is usually attributed to Nagaharu U, who produced the figure commonly known as the *Brassica* “U’s Triangle” or “Triangle of U” (U Nagaharu 1935). The six “U’s Triangle” species were found to comprise three diploid species, with genome complements $2n=2x=AA$ (*B. rapa*), $2n=2x=BB$ (*B. nigra*) and $2n=2x=CC$ (*B. oleracea*), and three allotetraploid species formed from additive genome combinations of the diploid species: $2n=4x=AABB$ (*B. juncea*), $2n=4x=AACC$ (*B. napus*), and $2n=4x=BBCC$ (*B. carinata*). Interestingly, Nagaharu U’s original figure also contained *Brassica napocampestris* (Frandsen and Winge 1932), $2n=6x=AAAACC$, an artificial hybrid between *B. napus* and *B. rapa* (previously referred to as *B. campestris*). These early cytogenetics studies involved very basic techniques: production of interspecific hybrids through cross-pollination and observations of chromosome number and chromosome behaviour in these hybrids at mitosis and meiosis. The ease in which many different species in the Brassicaceae hybridise, either through hand-pollination or via embryo rescue, protoplast fusion or other methods (FitzJohn et al. 2007), has provided a wealth of cytogenetic information. Pairing between chromosomes from different *Brassica* genomes was observed in haploids with genome complements A, B, C, AC, BC and AB, as well as in interspecific Brassicaceae hybrids of various types (Harberd and McArthur 1980) (Fig. 2.1). The propensity of chromosomes to associate at meiosis through regions of ancestral homoeology in the absence of meiotic control provides a crude but effective means of determining phylogenetic relationships (Mizushima 1980). Classical cytogenetics in *Brassica* and *Arabidopsis* has also been used in the identification of nuclear organisational regions (NORs) and provision of a basic karyotype using C-banding (Olin-Fatih and Heneen 1992; Koornneef et al. 2003).

The advent of molecular marker technologies initiated a decline in interest in cytogenetics, but modern molecular cytogenetics often provides a means of investigating regions of repetitive DNA which are otherwise recalcitrant to pure molecular studies, as demonstrated in studies of *Arabidopsis* centromere sequences (Murata et al. 1994; Heslop-Harrison et al. 1999). Molecular cytogenetics techniques have enabled further resolution of chromosomal relationships and genetic control of meiosis in the Brassicaceae, with numerous studies utilising genomic in situ hybridisation and more refined fluorescent in situ hybridisation techniques to confirm and expand upon these early findings.

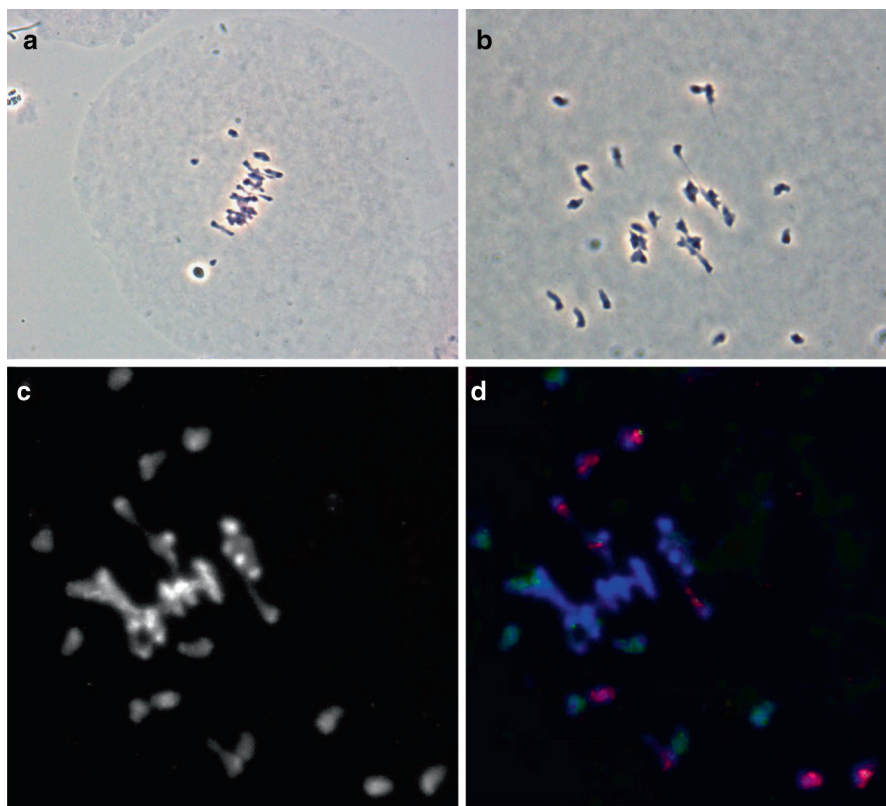


Fig. 2.1 Observations of meiosis in *Brassica* interspecific hybrids using classical cytogenetics and fluorescent in-situ hybridisation. (a) Metaphase I in a *B. juncea* × *B. carinata* hybrid ($2n=BBAC=35$), taken using 1 % acetic acid carmine stain with a phase contrast microscope and 1,000× magnification. (b) Metaphase I in a *B. juncea* × *B. napus* hybrid ($2n=AABC=37$), taken using 1 % acetic acid carmine stain with a phase contrast microscope and 1000× magnification. (c) Metaphase I in a *B. juncea* × *B. napus* hybrid ($2n=AABC=37$), taken using DAPI stain with a fluorescent microscope and 1,000× magnification. (d) Metaphase I in a *B. juncea* × *B. napus* hybrid ($2n=AABC=37$), taken using a fluorescent microscope and 1,000× magnification, after fluorescent in-situ hybridisation labelling of the B genome (green, fluorescein isothiocyanate), C genome (red, Texas Red) and background stain using DAPI (A genome, blue)

2.3 Fluorescent In Situ Hybridisation

Fluorescent in situ hybridisation (FISH) refers to the use of fluorescently-labelled DNA probes to bind (hybridise) to specific regions on chromosome spreads on microscope slides. Initially, the FISH protocol was based on the methods used previously for RFLPs, given the presumed similarity in the hybridisation protocol, but over the years many research groups have developed in-house modifications and optimisations. A particularly useful approach was taken in recent years: removing

each protocol step one by one and assessing if the protocol still worked, the end result of which was a greatly reduced FISH protocol (Kato et al. 2004). However, all FISH protocols involve basically the same process: firstly, the fluorescent labelling of the probe (usually by nick translation or random priming techniques, which insert fluorescently labelled nucleotides into the probe DNA sequence), secondly, the hybridisation of the probe to a denatured genomic chromosome spread on a microscope slide, and thirdly, visualisation of the probe label. Additional steps are mainly concerned with optimisation of the probe signal to background noise ratio, which involves steps such as removal of RNA using RNase enzyme treatment and washes with salt solutions at various temperatures. Denaturing the genomic DNA to allow probe binding may also be done through a number of different mechanisms, of which heat and treatment with formamide are the most common.

Genomic in situ hybridisation (GISH) is a special case of FISH whereby whole genomic DNA is used as a probe instead of specific DNA sequences. Early FISH studies used radioactive labels, but this has given way to use of fluorescent molecules (such as fluorescein isothiocyanate (FITC)) bound to antibodies such as avidin, biotin or digoxigenin as DNA probes, with a background chromosome stain of 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). In recent years the use of directly labelled fluorescent dNTPs, which can be integrated into the probe DNA directly using techniques such as nick translation, has also become popular in *Brassica* cytogenetics labs (Xiong and Pires 2011). The addition of fluorescent labels to chromosome spreads can result in a wealth of additional information (Fig. 2.1).

2.4 Chromosome Painting: Early Genomic and Fluorescent In Situ Hybridisation in the Brassicaceae

The earliest use of FISH in the Brassicaceae involved the mapping of repetitive DNA sequences (Iwabuchi et al. 1991) and rDNA loci (Maluszynska and Heslop-Harrison 1993). These and subsequent studies were useful in elucidating the number of rDNA loci (Snowdon et al. 1997a) and in presenting some information about the highly repetitive centromeric regions and conserved telomeric regions in *Brassica* (Hasterok et al. 2005). Molecular cytogenetics studies in model plant *Arabidopsis thaliana* have been highly informative in investigations of meiosis, repetitive DNA sequences and chromosome structure (Koornneef et al. 2003). In the agriculturally important *Brassica* genus, a common application of molecular cytogenetics involves the use of GISH to track chromosome introgressions through generations of backcrosses after interspecific hybridisation (Chen et al. 2011; Snowdon et al. 1997b; Navabi et al. 2011), and more recently the use of FISH probes to differentiate between more closely related genomes for the same purpose (Schelfhout et al. 2006). The ready hybridisation between species in the Brassicaceae has been utilised to effect transfer of useful genetic diversity or genes of interest into crops in breeding programs (Saal et al. 2004; Rygulla et al. 2007), and GISH allows these genomic introgressions to be tracked and analysed.

Another common use of GISH is to investigate meiotic pairing behaviour in interspecific hybrids, in order to assess genomic relationships and predict the success of genomic introgressions for crop improvement. Pairing between each of the genomes in the allotetraploid *Brassica* species *B. juncea* ($2n = AABB$), *B. napus* ($AACC$) and *B. carinata* ($BBCC$) has been observed using FISH and GISH (Mason et al. 2010), as has extensive pairing between *B. napus*, *B. oleracea* and *B. rapa* of different genotypes and ploidy levels (Leflon et al. 2006; Nicolas et al. 2008; Nicolas et al. 2009; Leflon et al. 2010). More distant relatives have also been noted using GISH to form chiasmatic associations at meiosis and recombinant chromosomes with *B. napus* or *B. rapa*, such as *Orychrophragmus violaceus* (Ma et al. 2006), *Isatis indigotica* (Tu et al. 2008), *Lesquerella fendleri* (Du et al. 2008) and *Capsella bursa-pastoris* (Chen et al. 2007).

FISH has also been used for the establishment of monosomic addition lines in the Brassicaceae, whereby a single chromosome from an alien genome is added to a known genomic complement (e.g. chromosome C1 of *B. oleracea* added to the ten chromosomes of the *B. rapa* A genome). This technique allows for the expression of genes belonging to a single linkage group to be analysed in the absence of other genomic loci. The production of C-genome addition lines in a *B. rapa* background allowed the complex trait of seed colour to be investigated (Heneen et al. 2012), as well as the pairing behaviour of individual homoeologous chromosomes. Trisomics and monosomics have also been greatly beneficial in *Arabidopsis* research (reviewed in (Koornneef et al. 2003)). However, the establishment of chromosome addition lines for the small, poorly-differentiated *Brassica* and *Arabidopsis* chromosomes necessitates the use of fluorescent in situ hybridisation. The process of backcrossing to eliminate all but one alien chromosome is not selective for a particular chromosome, and conclusively identifying which chromosome is present and confirming lack of chromosome fusions with the host genome can most readily be achieved by the use of FISH. GISH may also be used to label the alien genome if the alien chromosomes are not readily distinguishable from the host genome, or again to confirm that the alien addition chromosomes are not recombinants (Wang et al. 2006).

FISH can also be used to identify homoeologous non-reciprocal translocations and recombinant chromosomes in other systems, such as synthetic *B. napus* (Szadkowski et al. 2010). In the *Brassica* genus, resynthesis of allotetraploid *B. napus* ($2n = AACC$), *B. juncea* ($2n = AABB$) and *B. carinata* ($2n = BBCC$) from diploid progenitors *B. rapa* ($2n = AA$), *B. nigra* ($2n = BB$) and *B. oleracea* ($2n = CC$) is possible. However, synthetic *B. napus* is generally unstable due to the high degree of homoeology and hence meiotic interaction between the A and C genomes (Szadkowski et al. 2010), which are far more closely related to each other than either is to the B genome (Lagercrantz and Lydiat 1996; Parkin et al. 2003). Although the A and C genomes are known to associate at meiosis due to conservation of the ancestral relationships between the chromosomes, the degree of association is also mediated by genetic factors (Mason et al. 2010; Leflon et al. 2006; Nicolas et al. 2009). Fluorescent in situ hybridisation has been used successfully in the Brassicaceae to determine why particular meiotic behaviours occur, and what kinds of genetic and genomic factors underlie the meiotic process.

Notable successes in using fluorescent in situ hybridisation to investigate meiosis in the Brassicaceae include the discovery of a major gene affecting the number of crossovers during meiosis in *B. napus* (Jenczewski et al. 2003), the elucidation of mechanisms behind stable polyploid formation in *Arabidopsis* (Comai et al. 2003) and the development of an *Arabidopsis thaliana* karyotype (Armstrong and Jones 2003).

2.5 BAC-FISH and Fluorescently Labelled Antibodies

The field of molecular cytogenetics is still expanding. New techniques are constantly being developed in humans and model species (Figuroa and Bass 2010), and many of these have potential applications in the Brassicaceae family. In particular, fluorescently-labelled antibodies and BACs have been used in timing and observing protein expression and co-localization during meiosis and mitosis (Zhang et al. 2008) and molecular karyotyping (Koornneef et al. 2003; Xiong and Pires 2011) in the Brassicaceae. In 2011, a molecular karyotyping set consisting of a chromosome “paintbox” of BAC probes was released for *Brassica napus*, in the hopes of providing a generally applicable means for cytogenetic identification of each of the 19 *B. napus* chromosomes (Xiong and Pires 2011). This consisted of a set of three BAC probes and four DNA markers (45S, 5S, CentBrI and CentBrII). The BACs were selected from a pool of BACs generated from *B. rapa* sequence: two produced a set of signals across the *B. napus* chromosomes (Xiong and Pires 2011), and the third acted as a GISH-like marker for the C genome, hybridizing to a C-genome specific repetitive sequence in a similar fashion to the BAC BoB014O06 used previously for the same purpose (Alix et al. 2008). The 45S and 5S markers for ribosomal nucleolar organisational regions are well-characterized in *Brassica* and *Arabidopsis*, and added additional chromosome information. Lastly, the CentBrI and CentBrII probes bind to *Brassica* centromeric repeats: the two probes represent different repeats, and chromosomes in *Brassica* have CentBrI, CentBrII or both types of repeats. However, this toolkit has yet to be proven to be of wide use in *Brassica napus*, and may be susceptible to genotypic differences in hybridisation of BAC sequence probes.

FISH can also be used to integrate genetic maps (such as those generated by linkage mapping with molecular markers) with physical maps (such as those produced by classical karyotype observations). This can be done through the hybridisation of fluorescently labelled BACs containing known molecular marker sequence to chromosome spreads, and was successfully used to integrate the *B. oleracea* physical and genetic maps in 2002 (Howell et al. 2002) and the *B. rapa* physical and genetic maps in 2009 (Kim et al. 2009). Co-localization and ordering of FISH markers along single chromosomes can also be used to determine the relative position of difficult-to-map genomic regions and for fine-scale integration of physical and genetic maps, as has been demonstrated in chromosome A7 of *B. rapa* (Xiong et al. 2010).

2.6 Future Prospects

Molecular cytogenetics advances in other fields, particularly in the medical domain, offer a glimpse of a promising future for the Brassicaceae. In future, molecular cytogenetics may aid in genetic engineering, such as development of mini-chromosome platforms for transgene expression (Dhar et al. 2011), and full elucidation of the link between physical behaviour of chromosomes at mitosis and meiosis, DNA sequences and expression of proteins during cell division in the Brassicaceae. The increasingly wide-spread use of fibre-FISH, whereby hybridisation takes place on extended DNA fibres, now allows fluorescently-labelled clones to be separated at a resolution of only a few kB, rather than the few Mb allowed by conventional FISH (Walling and Jiang 2012). In addition, the *B. rapa* genome was released last year (Wang et al. 2011), and the imminent release of the *B. oleracea* and *B. napus* genomes will in upcoming years be joined by a larger set of wild Brassicaceae as well as the genomes of mustard species *B. juncea*, *B. carinata* and *B. nigra*. This unprecedented availability of sequence information will both facilitate the development of FISH probes and complement the ability of molecular cytogenetics to interrogate meiosis and meiotic behaviour. In addition, molecular cytogenetics offers a toolkit to aid in integration of physical and genetic maps, and in particular in addressing chromosome rearrangement, duplication and deletion events that are difficult to identify solely from sequencing or mapping information and are known to be prevalent in the complex ancestral and recent polyploid genomes of the Brassicaceae. From the birth of molecular cytogenetics in the Brassicaceae 20 years ago, technological developments and pioneering research studies have taken molecular cytogenetics in this family to exciting new heights, and the next 20 years of cytogenetics in the Brassicaceae promise to be equally eventful.

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Chapter 3

Distant Hybridization Involving Different In Vitro Techniques

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Abstract The Brassicaceae family has extensive genetic type and variation, which makes distant hybridization to be a potential strategy for integrating important traits such as quality and resistance to diseases and/or pests into the cultivated species from wild species. However, wide cross incompatibility, often leading to fail to obtain hybrids, limits the application of distant hybridization to crucifers' breeding. Nowadays, with the development of in vitro techniques, it is possible to use these genetic resources by distant hybridization. This chapter reviews the in vitro techniques including somatic hybridization, embryo rescue, microspore embryogenesis, and chromosome doubling that are involved in distant hybridization.

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