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Chromosome-based genomics in the cereals

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Abstract

The cereals are of enormous importance to mankind. Many of the major cereal species – specifically, wheat, barley, oat, rye, and maize – have large genomes. Early cytogenetics, genome analysis and genetic mapping in the cereals benefited greatly from their large chromosomes, and the allopolyploidy of wheat and oats that has allowed for the development of many precise cytogenetic stocks. In the genomics era, however, large genomes are disadvantageous. Sequencing large and complex genomes is expensive, and the assembly of genome sequence is hampered by a significant content of repetitive DNA and, in allopolyploids, by the presence of homoeologous genomes. Dissection of the genome into its component chromosomes and chromosome arms provides an elegant solution to these problems. In this review we illustrate how this can be achieved by flow cytometric sorting. We describe the development of methods for the preparation of intact chromosome suspensions from the major cereals, and their analysis and sorting using flow cytometry. We explain how difficulties in the discrimination of specific chromosomes and their arms can be overcome by exploiting extant cytogenetic stocks of polyploid wheat and oats, in particular chromosome deletion and alien addition lines. Finally, we discuss some of the applications of flow-sorted chromosomes, and present some examples demonstrating that a chromosome-based approach is advantageous for the analysis of the complex genomes of cereals, and that it can offer significant potential for the delivery of genome sequencing and gene cloning in these crops.

Introduction

The cereals (wheat, rice and maize *inter alia*) are a key component of human nutrition. Their cultivation provided self-sufficiency in food and animal feed, and this was instrumental in the establishment of all the major civilizations in Central America, the Middle East, and across Asia. Cereals remain the most important of the crops, whether measured by tonnage or by market value. Bread wheat is the single largest traded crop with a global annual production exceeding 600 million tons (http://www.fao.org/), equivalent to about 100 kg grain per capita. To secure cereal production in the face of a growing human population, and to maintain its yield and stability across environments threatened with climate change, efficient breeding for yield, quality and resistance to biotic and abiotic stress will be paramount. Genomics has the potential to make a significant contribution to these challenges, in that it can advance our knowledge of the molecular mechanisms underlying the determination of key quantitative and qualitative agronomic traits.

Taxonomically, all the cereals belong to the *Poaceae* (grass) family, distributed across four of its five main subfamilies: sorghum (*Sorghum bicolor*), maize (*Zea mays*), pearl millet (*Pennisetum glaucum*) and foxtail millet (*Setaria italica*) belong to the Panicoideae; finger millet (*Eleusine indica*) to the Chloridoideae; rice (*Oryza sativa*) to the Ehrhartoideae; and wheat (*Triticum* spp.), barley (*Hordeum vulgare*), oats (*Avena sativa*) and rye (*Secale cereale*) to the Pooideae (Table 1). The species differ markedly from one another in terms of chromosome number, ploidy level and genome size. As wheat, barley, oats and rye, and to a lesser extent maize, have very large nuclear genomes (Table 1), their chromosomes are particularly amendable to microscopic analysis and manipulation. The ability of the polyploids wheat and oats to tolerate aneuploidy has facilitated the development of a plethora of precise cytogenetic stocks, which include chromosome deletion lines, alien chromosome addition lines and substitution lines (Sears 1954, Islam *et al.* 1981, Joppa 1993, Jiang *et al.* 1994, Endo & Gill 1996, Riera-Lizarazu *et al.* 1996). These features have been exploited for gene location, genetic analysis, to analyse karyotype evolution and to understand genome structure.

Individual chromosomes were first identified using their relative length, arm ratio, presence of secondary constriction(s), and behaviour during meiosis. The introduction of differential staining – particularly Giemsa C-banding – allowed unambiguous chromosome identification and the characterization of structural changes (Linde-Laursen 1988; Gill *et al.* 1991). Molecular cytogenetics later provided even more precise tools to identify chromosomes and chromosome segments, and to unravel chromosome organization at the molecular level (Pedersen & Langridge 1997). Cytogenetic technologies and cytogenetic stocks continue to play an important role in the genomics era, for example by facilitating the physical mapping of molecular markers (Röder *et al.* 1998, Künzel *et al.* 2000, Kynast *et al.* 2004) and expressed sequences (EST) (Qi *et al.* 2004).

Large genomes and the polyploid state have stimulated much of recent progress in cytogenetics and genome analysis in the cereals, but in the genomics era these properties have become a liability. The sequencing of large genomes is capital-intensive, and the assembly of a genome sequence is greatly hampered, if not completely prohibited, by the presence of a significant proportion of repetitive DNA (up to 90% in the wheat genome, Paux *et al.* 2006). The simultaneous presence of at least two highly related genomes in the allopolyploids presents a further serious obstacle for sequence assembly. Thus many cereal genomes are not only considered intractable with respect to whole genome sequencing; the process of positional gene cloning is also made laborious and time-consuming.

In this review we survey the methods used for flow cytometry, and their application in the analysis of large and complex cereal genomes. Specifically, we describe how particular cytogenetic stocks can be exploited to dissect the genomes into their chromosome and chromosome arm components. Examples

Table 1. The major cereal crops in the Poaceae, their taxonomic classification and their genome characteristics

Species	Common name	Subfamily	Tribe	Chromosome number	Genome size (1C)	Reference
Oryza sativa L.	Rice	Ehrhartoideae	Oryzeae	2n = 2 × = 24	490 Mbp	Bennett & Smith 1976
Sorghum bicolour (L.) Moench	Sorghum	Panicoideae	Andropogonae	2n = 2 × = 20	809 Mbp	Laurie & Bennett 1985
Zea mays L.	Maize	Panicoideae	Andropogonae	2n = 2 × = 20	2793 Mbp	Doležel & Lucretti 1995
Eleusine coracana (L.) Gaertn.	Finger millet	Chloridoideae	Eragrostideae	2n = 4 × = 40	1593 Mbp	Bennett & Smith 1976
Pennisetum glaucum (L.) R.Br.	Pearl millet	Panicoideae	Paniceae	2n = 2 × = 14	2622 Mbp	Bennett & Smith 1976
Avena sativa (L.)	Oat	Poideae	Avenae	2n = 6 × = 42	12961 Mbp	Bennett & Smith 1976
Hordeum vulgare (L.)	Barley	Poideae	Triticeae	2n = 2 × = 14	5100 Mbp	Doležel *et al.* 1998
Secale cereale (L.)	Rye	Poideae	Triticeae	2n = 2 × = 14	7933 Mbp	Doležel *et al.* 1998
Triticum turgidum Desf. var. *durum*	Durum wheat	Poideae	Triticeae	2n = 4 × = 28	12030 Mbp	Bennett & Smith 1976
Triticum aestivum (L.)	Bread wheat	Poideae	Triticeae	2n = 6 × = 42	16979 Mbp	Bennett & Smith 1976

are presented to demonstrate that a chromosome-based approach provides a powerful tool for genome analysis in the cereal crops.

Flow cytometry of chromosomes

An array of techniques has been developed over the years to allow for the microscopic analysis of higher plant chromosomes. Common to all these diverse approaches is that the chromosomes must first be immobilized on a flat surface, usually a glass slide. This requirement imposes a significant limitation on throughput and hampers the manipulation of particular chromosomes. In contrast to microscopy, flow cytometry entails holding chromosomes in a liquid suspension, which allows them to be passaged in a fast-moving narrow stream (Doležel *et al.* 2005a). This has the clear advantage over slide-based techniques that the analysis can be automated, and therefore progressed at a rate ranging from several hundred to well over 1000 chromosomes per second. As the chromosomes pass through an intense and focused light beam they cause the light to be scattered, and the amount of scattered light (which is readily measurable) is correlated with the size of the chromosome which caused the scattering. The effect can be magnified by staining the chromosome suspension with a DNA-binding fluorochrome. Each light pulse is converted to an electric current pulse by a photomultiplier (a photodiode in case of forward-angle light scatter) and measurements made for pulse height (the maximum value of fluorescence intensity along the chromosome), pulse area (integrated fluorescence intensity) and pulse width (related to the length of chromosome).

A series of values describing the fluorescence and light-scattering properties is generated in real time for each chromosome measured. The analysis of several thousand chromosomes generates sufficient data to characterize the karyotype. The histogram of relative chromosome fluorescence intensities is referred to as the flow karyotype. Ideally, each chromosome is represented on a flow karyotype by a single peak, but this is not the case for most plant species, since their chromosomes are generally not well enough discriminated from one another in size (Doležel *et al.* 2004, see also Figures 1 and 2 for examples).

Difficulties in discriminating individual chromosomes may limit the application of flow karyotyping to analyse structural chromosome changes in plants. However, this limitation is in part compensated for by the potential of flow cytometry to physically isolate large numbers of certain specific chromosomes and/or groups of chromosomes. This sorting is effected at high speed (typically 5–30 chromosomes per second) so that purified preparations containing many copies of specific chromosomes can be generated efficiently. Flow cytometry is unique in its ability to concentrate single chromosomes in sufficient numbers, since neither microdissection nor laser capture (Potz *et al.* 1996, Stein *et al.* 1998) is able to practically generate more than a few tens of copies of any particular chromosome.

The development of flow cytogenetics for cereals

The use of flow cytometry for both chromosome analysis and sorting has been termed flow cytogenetics. Following its successful application in human and animal genetics and genomics, relevant methods have been adapted for a range of plant species (reviewed by Doležel *et al.* 2004). The first flow karyotyping of a cereal species was published by Wang *et al.* (1992), who analysed chromosomes isolated from a bread wheat cell suspension culture. Hydroxyurea (a DNA synthesis inhibitor) was used to synchronize the cell division cycle, and colchicine (a mitotic spindle poison) was applied to block the cells at mitotic metaphase.

Enzymatic hydrolysis then converted these cells to protoplasts, and mitotic chromosomes were released into solution by hypotonic lysis and mechanical shearing. The resulting chromosome suspensions were stained simultaneously with Hoechst 33258 and Chromomycin A3 fluorochromes, which bind preferentially to, respectively, AT- and GC-rich DNA regions. The resulting biparametric flow karyotypes comprised a number of peaks, but it was not clear whether these peaks represented one or more chromosomes (Wang *et al.* 1992), nor was the level of contamination within the peaks due to chromosome clumps and fragments determined. Nevertheless, some 100 000 chromosomes were isolated from one peak and used to construct a genomic DNA library. An analysis of randomly selected clones from this library revealed an enrichment for chromosome 4A. Although only 300 recombinant clones were obtained, this first chromosome sorting in cereals demonstrated the potential of flow cytogenetics in wheat.

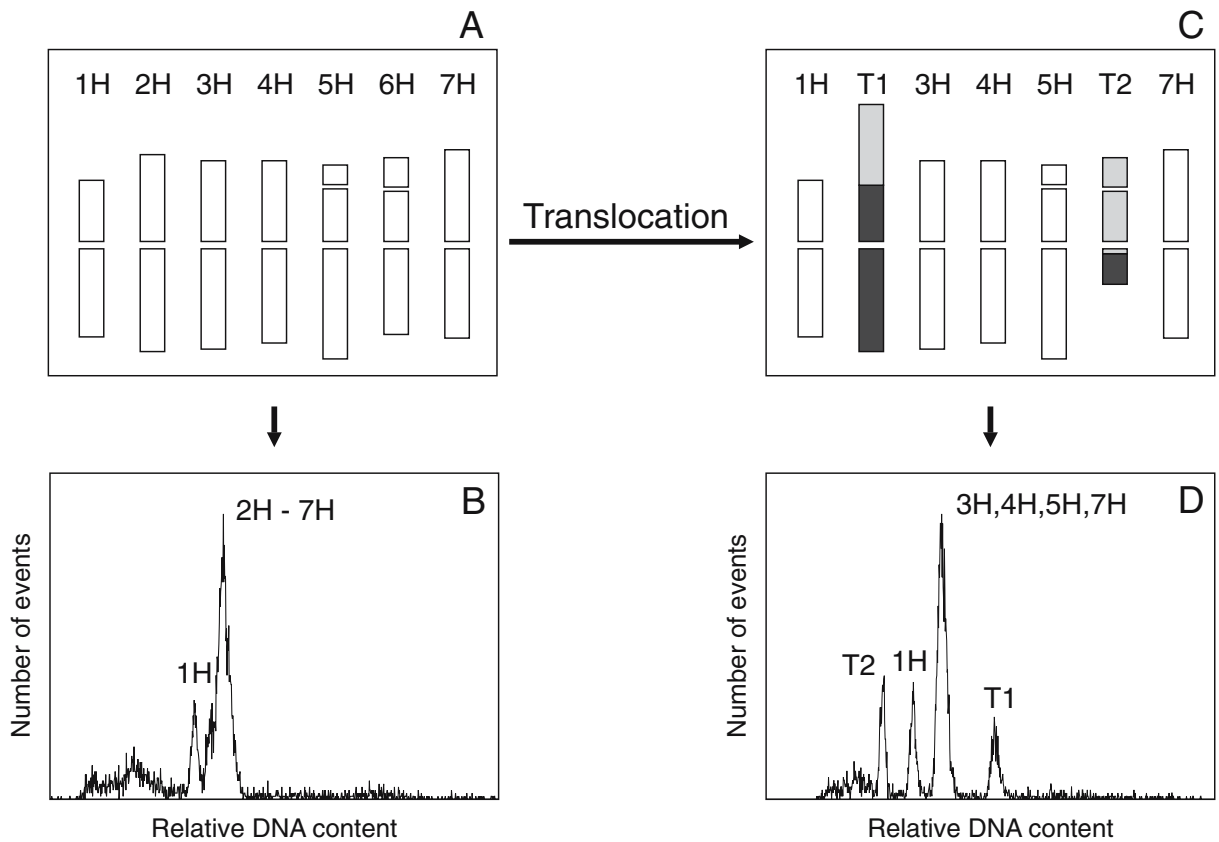

Figure 1. Alterations in the flow karyotype of barley in response to changes in chromosome size. **A**: In the wild-type karyotype the barley chromosomes, apart from chromosome 1H, differ from one another only marginally in length and hence also DNA content. **B**: The flow karyotype of wild-type barley, in which only the chromosome 1H peak is well separated. The remaining six chromosomes form a composite peak and cannot be discriminated from one another. **C**: In barley line T2-6y, a reciprocal translocation results in a large T6HL-2HS·2HL (T1), and a small T6HS·6HL-2HS (T2) chromosome. **D**: The relative DNA contents of the two translocation chromosomes differ from that of other five chromosomes and both are well separated on a flow karyotype.

The outcome of the Wang *et al.* (1992) study highlighted some of the problems affecting similar work in other crops (reviewed by Doležel *et al.* 1994). Plant cells cultured in liquid suspensions are characterized by genetic instability. The cell line used by Wang *et al.* was later cytogenetically analysed by Schwarzacher *et al.* (1997), who showed it to be heterogeneous and affected by both aneuploidy and structurally aberrant chromosomes. Chromosomal heterogeneity hinders the induction of a high level of mitotic synchrony. Furthermore, the isolation of chromosomes from protoplasts requires a period during which chromosomes tend to split into sister chromatids and eventually de-condense. Although biparametric analysis using dyes differing in base-pair preference is a standard method in animal and human flow cytogenetics, in plants it does not appear to improve chromosome discrimination over what is achieved with monoparametric analysis. The most probable reason is the prevalence of dispersed repeats in plant genomes, resulting in small differences in overall AT/GC ratios among individual chromosomes (Lucretti & Doležel 1997).

Further progress in flow cytogenetics of cereals has relied on methodological improvements achieved in other species. At first a novel protocol for chromosome isolation was introduced (Doležel *et al.* 1992), according to which chromosome suspensions were prepared by the mechanical homogenization of formaldehyde-fixed root tips. A mild fixation with formaldehyde prior to homogenization increased the yield of intact chromosomes. This protocol, now almost universally used in plant flow cytogenetics (Doležel *et al.* 2005a,b), has as its major advantages the easy handling of seedlings during the cell synchronization pro-

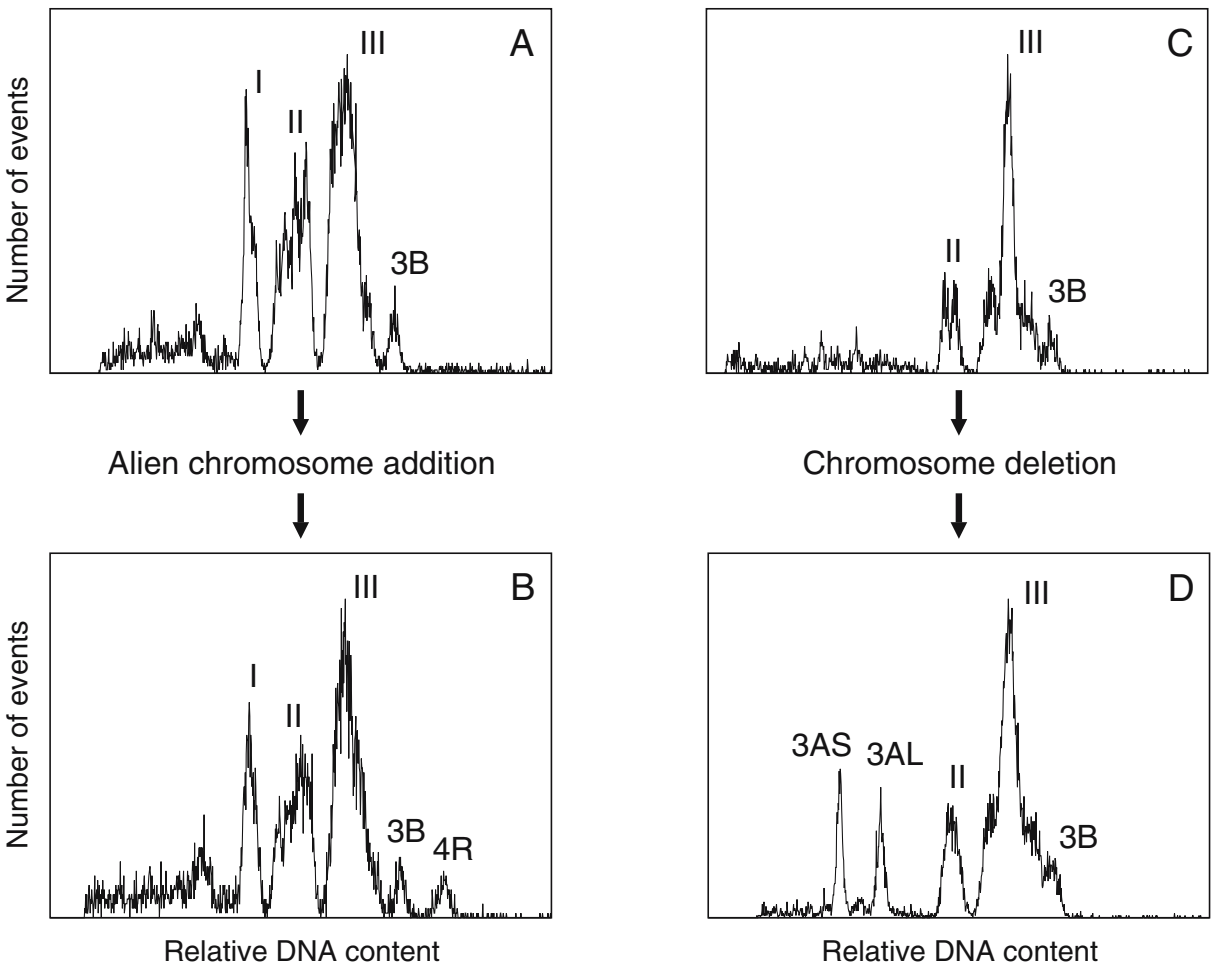

Figure 2. Changes in the flow karyotype of aneuploids, relative to that of euploid wheat. **A**: The flow karyotype of euploid bread wheat consists of the chromosome 3B peak, the small composite peak I containing chromosomes 1D, 4D and 6D, and the two large composite peaks II and III containing the remaining 17 chromosomes. **B**: The flow karyotype of a bread wheat line carrying an added pair of rye chromosome 4R. The peak representing 4R is clearly separated from the wheat chromosomes. **C**: The flow karyotype of euploid durum wheat consists of three peaks: chromosome 3B, a small composite peak II representing chromosomes 1A and 6A, and a large composite peak III representing the remaining 11 chromosomes. **D**: The flow karyotype of double ditelosomic 3A in durum wheat is characterised by additional peaks representing the short arm telosome 3AS and the long arm telosome 3AL.

cedure, a high degree of synchrony, and karyological stability. The mechanical isolation eliminates the need for enzymatic hydrolysis, and the fixation both arrests the cells at the desired stage (metaphase), and makes the chromosomes less liable to mechanical shearing during the isolation, analysis and sorting processes. The second methodological improvement was pioneered by Lucretti *et al*. (1993) in an analysis of cytogenetic stocks of broad bean (*Vicia faba* L.) in which chromosome lengths had been altered by translocations. A directed choice of translocation lines allowed for individual chromosomes to be discriminated by monoparametric flow karyotyping (Doležel & Lucretti 1995). As all other attempts to improve discrimination by flow cytometry had failed, including the use of biparametric staining (Lee *et al*. 2000, Lucretti & Doležel 1997, Kovářová *et al*. 2007) and fluorescent labelling of repetitive DNA sequences (Macas *et al*. 1995), the use of cytogenetic stocks, including translocations, deletions and alien additions, has emerged as the best approach to identify and sort particular chromosomes and their arms (Doležel *et al*. 2004). In the following, we analyse the development of flow cytogenetics in the Triticeae, and in maize and rice.

Flow cytogenetics in the Triticeae

In a modification of the Doležel protocol for barley (Lysák *et al.* 1999), an average yield of 5×10^5 chromosomes from 50 root tips was achieved. High-resolution flow karyotypes were obtained from DAPI-stained chromosome suspensions. Despite this, only chromosome 1H could be discriminated in the flow karyotype, with the remaining six chromosomes (2H–7H) forming a composite peak (Figure 1A, B). However, in some cultivars, a chromosome 2H peak was also resolved (Lee *et al.* 2000, Suchánková *et al.* 2006). Inspired by the work of Lucretti *et al.* (1993), Lysák *et al.* (1999) then studied a series of barley lines in which the length of some chromosomes was altered due to reciprocal translocations. At least three chromosomes could be recognized in the flow karyotypes of particular cytogenetic stocks (Figure 1C, D). The purity of the preparations of sorted chromosomes reached 97%, as determined by a microscopic analysis of sorted chromosomes labelled by the PRINS (primed *in-situ* labelling) technique using primers recognizing GAA microsatellite motifs. The small degree of contamination was due to the rare presence of chromosome arms and chromatids of other chromosomes. Lysák *et al.* (1999) were also able to demonstrate the utility of sorted translocation chromosomes for the physical mapping of DNA sequences at the sub-chromosomal level.

A modification of Doležel's root tip method for the preparation of bread wheat chromosome suspensions was reported by Lee *et al.* (1997). As the formaldehyde fixation step was omitted, a rather low yield of 82 000 chromosomes from 60 root tips was obtained (Gill *et al.* 1999). Propidium-iodide stained material produced flow karyotypes in which peaks assumed to represent chromosomes and chromatids could be resolved, but no attempt was made to identify the chromosome content of any of the individual peaks. The authors contrasted chromosomes from the wheat cultivar 'Chinese Spring' with those from a line of 'Chinese Spring' lacking chromosome arm 1DL (ditelosomic 1DS). Flow karyotypes of the latter included a peak absent in the former, assumed to be composed of the 1DS telosome, and which could be sorted with a purity exceeding 82% (Gill *et al.* 1999). This observation confirmed the utility of cytogenetic stocks to identify particular chromosomes. A year later, Vrána *et al.* (2000) reported high-resolution flow karyotyping in bread wheat, and were able to assign all 21 wheat chromosomes to four peaks of a flow karyotype, using a combination of PRINS on sorted chromosomes with primers targeted to GAA microsatellites and *Afa* repeats, and chromosome-specific PCR. The largest wheat chromosome (3B) formed a discrete peak and was readily sortable. The remaining 20 chromosomes clustered into the three other peaks, one of which consisted of chromosomes 1D, 4D and 6D (Figure 2A). In addition to describing the bread wheat flow karyotype, flow karyotyping was also shown to be useful for the detection of translocations between wheat chromosomes.

The flow karyotypes of a set of 58 varieties and landraces of bread wheat demonstrated the reproducibility of the technique, and were used to detect structural chromosome changes and chromosome polymorphisms (Kubaláková *et al.* 2002). The wheat/rye translocation 1BL·1RS is present in many modern wheat cultivars and its presence generates a diagnostic change in flow karyotype. However, the discriminating peak was not sufficiently resolved to allow effective sorting of the translocated chromosome. Other translocation chromosomes, in particular 5BL·7BL and 4AL·4AS-5BL, produced flow karyotype peaks which were discrete enough to permit sorting. However, the most important breakthrough was that almost all the wheat telosomes (with the exception of 3BL and 5BL), which are maintained in stable cytogenetic stocks covering most of the 42 chromosome arms (Sears 1954), could be recognized against the background of the rest of the genome, and sorted. The 3BL and 5BL arms were isolated from stocks carrying them as isochromosomes.

The flow karyotype of tetraploid (durum, or macaroni) wheat was characterized by Kubaláková *et al.* (2005). Due to the absence of D-genome chromosomes, only three peaks were generated, one containing chromosome 3B, a small peak (1A and 6A), and a large peak containing the remaining 11 chromosomes (Figure 2C). Assignment of chromosomes to flow karyotype peaks was achieved by applying FISH (fluorescence *in-situ* hybridization) to sorted chromosomes using probes targeting the GAA microsatellite motif, *pSc*119.2 and *Afa* repeats. Although durum wheat has a smaller number of chromosomes than bread wheat (14 against 21), only one chromosome (3B) could be unequivocally sorted. Nonetheless, by exploiting the double ditelosomic lines of durum wheat, in which each chromosome in turn is represented by a pair of telosomes (Joppa 1993), it was possible to isolate each

A- and B-genome chromosome arm (Kubaláková *et al*. 2005, and Figure 2D). As the flow karyotype of durum wheat was less complex than that of bread wheat, the telosome peaks were better defined, which in turn resulted in the recovery of a 90–98% purity level in sorted preparations.

Rye has the largest nuclear genome (1C ~ 7.9 Gbp) among the diploid Triticeae species. Methods for the preparation of chromosome suspensions and flow karyotyping were developed by Kubaláková *et al*. (2003). The karyotypes consisted of a composite peak representing chromosomes 2R–7R. Chromosome 1R formed a shoulder on the left side of the composite peak in most cultivars. However, in some cultivars (e.g. 'Imperial'), the 1R peak was fully resolved and the chromosome could be sorted. Interestingly, the flow karyotype of rye was similar to that of barley, whose nuclear genome is somewhat smaller (1C ~ 5.1 Gbp). This implies that, during the speciation of these crop species, each chromosome expanded in a proportional fashion. Flow karyotyping was also able to detect both inter-chromosome translocations and the presence of supernumerary B chromosomes.

Although direct flow sorting could only successfully isolate a single rye chromosome, recourse to wheat–rye chromosome addition lines overcame this limitation. In these lines each pair of rye chromosomes in turn is present in a bread wheat background, and the added rye chromosomes 2R–7R are sufficiently different in size to any of the wheat chromosomes to allow for their straightforward identification and sorting (Kubaláková *et al*. 2003, and Figure 2B). The sensitivity of the profiles was such that the dosage of the added rye chromosome in a population could be deduced from its flow karyotype based on relative height of the rye peak. More recently, Šafář *et al*. (2006) exploited a wheat–rye ditelosome addition line to purify chromosome arm 1RS. The same approach could be used to sort independently those chromosome arms of rye, which are shorter than chromosome 1D, the shortest in the bread wheat complement.

Of the intact barley chromosomes, to date only chromosome 1H, and in certain genotypes chromosome 2H, could be flow-sorted (Figure 1). The success of the alien addition line approach for rye chromosomes stimulated Suchánková *et al*. (2006) to explore a similar strategy in barley. Each of the chromosome arms of barley present in the wheat–barley ditelosomic addition lines was sortable, although no attempt was made to sort the arms of 1H, as this chromosome can be isolated directly from the barley flow karyotype. In any case, given the sterility of the telosomic 1HL addition (Islam & Shepherd 1990, 2000), only the short arm of 1H would have been accessible via this route.

A flow cytogenetic toolbox for the Triticeae

Strategies have now been developed to dissect the genomes of the major Triticeae crops into their individual chromosomes and chromosome arms (Doležel *et al*. 2005b), relying on the availability of appropriate wheat cytogenetic stocks and alien chromosomal structural variants. As shown in Table 2,

Table 2. The possibilities for dissecting the nuclear genomes of the Triticeae crops by flow sorting

Karyotype	Chromosomes and chromosome arms that can be isolated by flow sorting*			
	Barley	Rye	Durum wheat	Bread wheat
	1C ~ 5.1 Gbp	1C ~ 7.9 Gbp	1C ~ 12 Gbp	1C ~ 17 Gbp
Wild-type	1H (12.2%)	1R (12.7%)	3B (8.6%);	3B (5.9%);
Telosomic lines	–	–	All arms (2.1–5.2%)	All arms, except 3BL, 5BL (1.3–3.2%);
Isochromosome lines	–	–	–	3BL, 5BL (3.3%, 3.4%)
Wheat chromosome addition lines	–	2R–7R (13.2–16.6%)	–	–
Wheat telosome addition lines	Arms of 2H–7H (5.9–9.0%)	1RS (5.6%)	–	–

* In some genotypes additional chromosomes can be isolated. The values in parentheses show the relative portions of the nuclear genome represented by the smallest and the largest chromosomes and/or chromosome arms that can be sorted. Relative chromosome (arm) lengths were taken from Marthe & Künzel (1994) for barley, Schlegel *et al*. (1987) for rye, Venora *et al*. (2002) for durum wheat and Gill *et al*. (1991) for bread wheat.

the Triticeae genomes can be dissected in this way into segments as small as 1.3% of the total genome, translating to about 220 Mbp in bread wheat or about half of the size of the fully sequenced rice genome (IRGSP 2005; Goff *et al*. 2002; Yu *et al*. 2002). Should segments smaller than chromosome arms be needed, it is possible to generate these via the activity of gametocidal chromosomes (Shi & Endo 1997, Yoshino *et al*. 1998). Thus, it may be possible in the future to isolate, via flow sorting, portions of the Triticeae genomes smaller than a chromosome arm.

Flow cytogenetics of maize

Lee *et al*. (1996) were the first to report a flow cytometric analysis of the chromosomes of maize. Chromosome suspensions were prepared from root meristems using a method modified from that of Doležel *et al*. (1992). Flow karyotypes of propidium-iodide stained samples matched the profile predicted from relative chromosome sizes, and suggested the possibility of discriminating and sorting two (chromosomes 1 and 10) of the 10 maize chromosomes. However, no attempt was made to assign chromosomes to flow karyotype peaks. Later, flow karyotypes of a range of maize inbred and hybrid lines were produced (Lee *et al*. 2002), revealing variation in the number and position of chromosome peaks, which presumably reflects different genome sizes and the presence of heterochromatic chromosomal knobs. At least five different chromosome types could be discriminated and sorted from five different maize lines, although confirmation of the purity of these preparations has not yet been forthcoming. The difficulties experienced in sorting the maize chromosomes prompted the exploration of an alternative strategy, particularly focusing on the oat–maize chromosome addition lines developed by Riera-Lizarazu *et al*. (1996). In these lines a single maize chromosome or chromosome pair is present in a hexaploid oat background. The oat–maize chromosome-9 addition line produced a flow peak corresponding to maize chromosome 9 and the chromosome was sorted at a level of purity exceeding 90% (Li & Arumuganathan 2001).

Flow cytogenetics of oats

In order to use an oat–maize addition line, Li & Arumuganathan (2001) had first to optimize a protocol for the preparation of chromosome suspensions from oat root tips. Although a flow karyotype of hexaploid oats cv. 'Starter-1' has been presented, it has not been clarified whether this allows for the sorting of any individual oat chromosomes. Thus, at present the potential of flow cytogenetics for dissecting the genome of oats remains to be assessed.

Flow cytogenetics of rice

Rice has a small genome, which has proved to be amenable to sequencing and gene cloning. Recently, the genomes of the two rice subspecies *indica* and *japonica* have been separately sequenced (Goff *et al*. 2002, Yu *et al*. 2002, IRGSP 2005), and rice is universally considered to be the appropriate model for cereal genomics (Xu *et al*. 2005). Lee & Arumuganathan (1999) optimized a protocol for isolating chromosomes from synchronized rice root tips. Although peaks thought to represent individual chromosomes were observed, the low resolution achieved prevented an estimation of how many, if any, chromosomes could be sorted. However, even a predicted flow karyotype showed that only one chromosome could be discriminated (Lee & Arumuganathan 1999). The rapid progress made in rice genomics makes flow cytogenetics unlikely to play an important role in rice compared to that in the large genome cereals.

The integration of flow cytogenetics and genomics

The sequencing of higher plant genomes is advancing rapidly and it is expected that 12 plant genomes will have been sequenced by 2006 (Paterson 2006, see also http://www.jgi.doe.gov). Candidates for sequencing are chosen largely on the basis of (small) genome size in order to avoid technical problems associated with the sequencing of complex genomes. For the latter, a strategy of sample sequencing has been proposed (Paterson 2006). Although this represents a large cost saving over whole genome shotgun sequencing, it suffers from the drawback that short DNA sequences cannot be readily assembled to reconstruct the full genome. Assembly becomes possible only when the sample sequences can be anchored to an extant physical genome map (Meyers *et al*. 2004).

Several methods have been applied to create physical maps, in which the genome is described by

an assembly of adjacent or overlapping segments. Two in particular of these are worth mentioning in the context of flow cytometry, although neither has been applied on any large scale to date. HAPPY mapping (Thangavelu *et al*. 2003) relies on the co-segregation of markers on randomly sheared genomic DNA fragments, while optical mapping (Aston *et al.* 1999) arrays digested DNA molecules and seeks to derive ordered restriction maps from images of the digested molecules. High molecular weight DNA is required for both these methods, and this can be prepared from flow-sorted cereal chromosomes. Thus sorted chromosomes could represent the basis of physical maps related *a priori* to a specific chromosome or chromosome arm.

Almost all genome-wide physical maps have been generated by the ordering of BAC (bacterial artificial chromosome) clones, via DNA fingerprinting (Luo *et al*. 2003). These maps serve to anchor short sequences to a genome scaffold, enable clone-by-clone sequencing and positional gene cloning. The assembly of physical maps in the large genome cereals is hampered by the large number of clones required to achieve full genome coverage, by the ubiquity of dispersed repeats, and by polyploidy. Thus the 9× coverage BAC library of bread wheat consists of 1.2×10^6 clones (Allouis *et al*. 2003) and although it is technically possible to fingerprint such a large numbers of clones, the assembly of reliable contigs and the construction of a physical map represents an enormous challenge.

Chromosome-specific BAC libraries

An elegant solution to the size problem is to create BAC libraries specific for small and defined genome segments, and this represents an area in which chromosome sorting can play a crucial role. Until 2004 it was unclear whether large-insert DNA libraries could be constructed from flow-sorted chromosomes, and three critical developments were seen as necessary to achieve this goal. These were: (1) the ability to sort individual chromosomes/chromosome arms in sufficiently large quantities, as discussed above; (2) the preparation of unsheared DNA from sorted chromosome preparations in a form suitable for BAC cloning (Šimková *et al*. 2003); and (3) the development of an efficient protocol for BAC cloning (Chalhoub *et al*. 2004). Thus, a subgenomic BAC library specific for bread wheat chromosomes 1D, 4D and 6D was generated by Janda *et al*. (2004), and one for bread wheat chromosome 3B by Šafář *et al*. (2004) (Table 3). The 3B library was constructed from a sample of 1.8×10^6 flow-sorted chromosomes and represents the first BAC library to be produced from a specific eukaryotic chromosome.

Most of the wheat and barley chromosomes can only be sorted as chromosome arms, rather than as whole chromosomes. As the arms represent smaller parts of the genomes than chromosomes, they must be sorted in higher numbers to provide enough DNA for BAC library construction. A 1BS BAC library has been generated recently from 6×10^6 purified chromosome arms, sorted from ditelosomic 1BS, a line lacking chromosome arm 1BL (Janda *et al*. 2006). Representing just 1.9% of the wheat genome (Table 3), the library achieves a 14.5 × coverage of 1BS. An even deeper (17×) coverage was generated in a 1RS BAC library (Šafář *et al*. 2006). This chromosome arm is present in many modern wheat cultivars in the form of a 1BL·1RS chromosome, as its presence is associated with improved agronomic performance. Notably, good coverage is achieved in these chromosome (arm) BAC libraries from a relatively modest number of clones (typically $< 10^5$), making the libraries practical to maintain and screen.

Table 3. BAC libraries already constructed from flow-sorted chromosomes of *Triticeae* species

	Chromosome			BAC library			
Species	Chromosome number	Genome fraction	Molecular size (Mb)	Number of clones	Mean insert size (kb)	Coverage	Reference
T. aestivum	1D, 4D, 6D	11.6%	1969	87 168	85	3.4×	Janda *et al*. (2004)
T. aestivum	3B	5.9%	995	67 986	103	6.2×	Šafář *et al*. (2004)
T. aestivum	1BS	1.9%	315	65 280	82	14.5×	Janda *et al*. (2006)
S. cereale	1RS	5.6%	442	103 680	83	17.0×	Šafář *et al*. (2006)
T. aestivum	3AS	2.1%	361	55 296	80	12×	Šimková *et al*. (in preparation)

The utility of subgenomic BAC libraries

About 10 Mb of wheat A-, B- and D-genome BAC sequence have been sequenced and analysed to date (Paux *et al.* 2006). However, most of this sequence relates to short segments (mean of about 200 kb of contiguous sequence) and originates mostly from gene-rich and telomeric regions of the genome. Thus it provides a rather limited and unrepresentative picture of the overall genome. Wheat's large genome size and the presence of three homoeologous genomes have hindered large-scale and representative analyses to date. A suggested means to generate sufficient random sequence to allow an unbiased assessment of genome sequence content and organization is target BAC end sequences (BES). Thus in maize, Messing *et al.* (2004) produced 475000 BES, equivalent to 307 Mb or 12% of the total genome. To achieve a similar representation in bread wheat, 3.3 million BES would be needed, and it would also be necessary to determine the chromosomal origin of each sequence in order to identify features specific to each of the constituent genomes. Alternatively, on the assumption that the genomes remained unchanged during the process of polyploidization, the three donor species could be analysed separately. However, as dramatic rearrangements are thought to have occurred following polyploidization (Feldman & Levy 2005), the ancestral genomes probably represent at best only an approximate model of the hexaploid genome, not mentioning difficulties in identifying the B-genome donor. Therefore, the most efficient means to expose the composition and evolution of the homoeologous genomes will be to analyse the individual chromosomes of bread wheat, using BAC libraries from sorted chromosomes.

The power of such a chromosome-based approach is well illustrated by current investigations of wheat chromosome 3B. The 67 968 BAC clones of the 3B BAC library have now been fingerprinted and assembled to construct a physical map of the chromosome (unpublished data). In the first phase of this assembly, Paux *et al.* (2006) generated 3306 contigs, which were used to select clones for BAC end sequencing and the characterization of chromosome structure. In all, 10 752 clones were sequenced, generating about 11 Mb of sequence evenly distributed along the chromosome. This sequence comprised 86% repetitive elements, 1.2% coding sequence (corresponding to 6000 genes) and 13% unknown sequence. A number of novel repetitive sequences was also uncovered. By means of a comparison with 2.9 Mb of random sequence from *Aegilops tauschii*, the D-genome donor, it appears that the large size of the B genome chromosomes is due to their higher content of repetitive elements. The transposable element (TE) families most likely responsible for the evolutionary differential expansion of the wheat genomes were also identified (Paux *et al.* 2006). Clearly, the availability of a purified preparation of a single chromosome was critical to reveal these features of the bread wheat B genome, results hard to achieve by performing an analysis at the whole genome level.

Perspectives in genome sequencing and gene cloning using subgenomic BAC resources

The establishment of a physical map of the bread wheat genome represents the first step towards the goal of sequencing the genome. In addition, a physical map of each of the 21 wheat chromosomes will serve to accelerate efforts to isolate, using a map-based approach, the hundreds of genes and QTL that have been genetically mapped to date. One of the most time-consuming steps in such cloning projects remains the establishment of a physical contig spanning the target. Chromosome-specific BAC libraries should allow a rapid drafting of the relevant physical map with contigs ranging from 200 kb to > 1 Mb. Once the target region has been identified on a high-density genetic map it is a straightforward procedure to then identify the candidate BAC contig(s), establish contigs spanning the gene of interest, and sequence the region to identify candidate genes. Thus the International Wheat Genome Sequencing Consortium (IWGSC, www.wheatgenome.org) plans now to construct chromosome arm-specific BAC libraries from cv. 'Chinese Spring' for which a genomic BAC library is also available (Allouis *et al.* 2003). If a particular allele of interest is not present in 'Chinese Spring', a pooled BAC library can be rapidly produced (Isidore *et al.* 2005) from a genotype carrying the trait of interest and screened with probes extracted from the 'Chinese Spring' physical map.

The targeted isolation of molecular markers

In addition to the construction of physical maps, other genomics applications of flow-sorted chromosomes deserve attention. Fine-scale genetic mapping requires the saturation of the genetic maps in the

region of the target, and thus the development of many localized molecular markers. Such markers can be retrieved from chromosome-specific DNA libraries constructed from just hundreds or thousands of chromosomes, which are then amplified either by DOP-PCR (Telenius *et al.* 1992) or by whole genome amplification with DNA polymerase phi29 (Dean *et al.* 2001). This strategy has been explored in both non-cereal crops (Arumuganathan *et al.* 1994, Požárková *et al.* 2002, Román *et al.* 2004) and rye (R. Kofler, personal communication). The major advantage of a chromosome-based approach is that the chromosomal specificity of each candidate marker can be verified, prior to genetic mapping, by a simple PCR from a template of flow-sorted chromosomes (Požárková *et al.* 2002). A further option for the targeted isolation of markers from chromosome-derived DNA is offered by DArT technology (Wenzl *et al.* 2004), based on microarray hybridization and designed to detect and type DNA variation at several hundred genomic loci in parallel. Although to date DArT has only been applied on genomic DNA, there is no reason to doubt that it would function equally well on a target of chromosomal DNA.

Effective markers can also be developed from BES. Paux *et al.* (2006) combined this idea with a chromosome-based approach in order to saturate the genetic map of wheat chromosome 3B, specifically targeting ISBP (insertion site-based polymorphisms) markers. Junctions between TE and non-TE sequences, which represent the site of insertion of a TE, were identified from 20 000 BES produced from the chromosome 3B BAC library. From 2000 of these junctions, which have proven to be highly polymorphic, 58 primer pairs were designed to assess the potential of ISBP for mapping in wheat. About 67% of these were assignable to bins defined by the standard wheat deletion line set, while the remainder were informative in various segregating populations. By extrapolation therefore, as many as 1300 BES-derived markers could be added to the chromosome 3B map. A similar approach is being used to saturate the genetic map of rye chromosome 1R, focusing on microsatellite sequence markers (Bartoš *et al.*, in preparation).

The molecular organization of cereal chromosomes

Localization of DNA sequences on chromosomes is commonly performed by FISH or PRINS to mitotic metaphase spreads from root tip meristems. However, practical considerations limit the number of analysable metaphase plates and so restrict the analytical throughput achievable. Furthermore, the sensitivity and specificity of FISH and PRINS are often compromised by interference from cell wall and cytoplasmic debris. The application of FISH and PRINS to sorted chromosomes (Lucretti *et al.* 1993, Kubaláková *et al.* 2001, Li & Arumuganathan 2001) provides the opportunity to screen hundreds of chromosomes per slide, with better resolution, sensitivity and throughput. In an analysis of flow-sorted rye B chromosomes, Kubaláková *et al.* (2003) were able to demonstrate rare translocations between A and B chromosomes. As these occurred at a frequency of just 0.5%, their discovery by conventional screening of metaphase squashes would have required the processing of hundreds of slides. FISH-based analyses of sorted wheat chromosomes have allowed for both the uncovering of polymorphism in GAA banding patterns (Kubaláková *et al.* 2002), and the construction of a molecular karyotype of durum wheat (Kubaláková *et al.* 2005).

The ability of FISH to resolve two adjacent DNA sequences depends on a combination of genome size and the degree of chromosome condensation. The minimum range of resolution is from 5–10 Mb for mitotic metaphase chromosomes, 0.2–1 Mb for meiotic pachytene chromosomes and 1–5 kb for stretched DNA fibres (de Jong *et al.* 1999). For some applications the resolution of the latter is higher than necessary but the resolution of pachytene FISH is too low, especially in large genome cereals, and particularly in polyploid species. Thus there is a need for a method which can provide an intermediate level of resolution. This was achieved by Valárik *et al.* (2004), who were able to extend, by up to 100×, the length of flow-sorted barley, rye and wheat chromosomes. The application of FISH to these stretched chromosomes gave a spatial resolution of up to 70 kb.

Chromosome painting is a technique central to human cytogenetics. Its principle is to label each chromosome in a specific manner, using FISH with a hybridization cocktail of many probes. A critical factor for the success of this technique is probe specificity. Despite many attempts to develop painting in large genome plants, it has been difficult to prevent cross-hybridization of probes, even though where they have been extracted from microdissected chromosome arms (e.g. wheat chromosome 5BL) or sub-arm segments (e.g. the distal quarter of the long arm of barley trans-

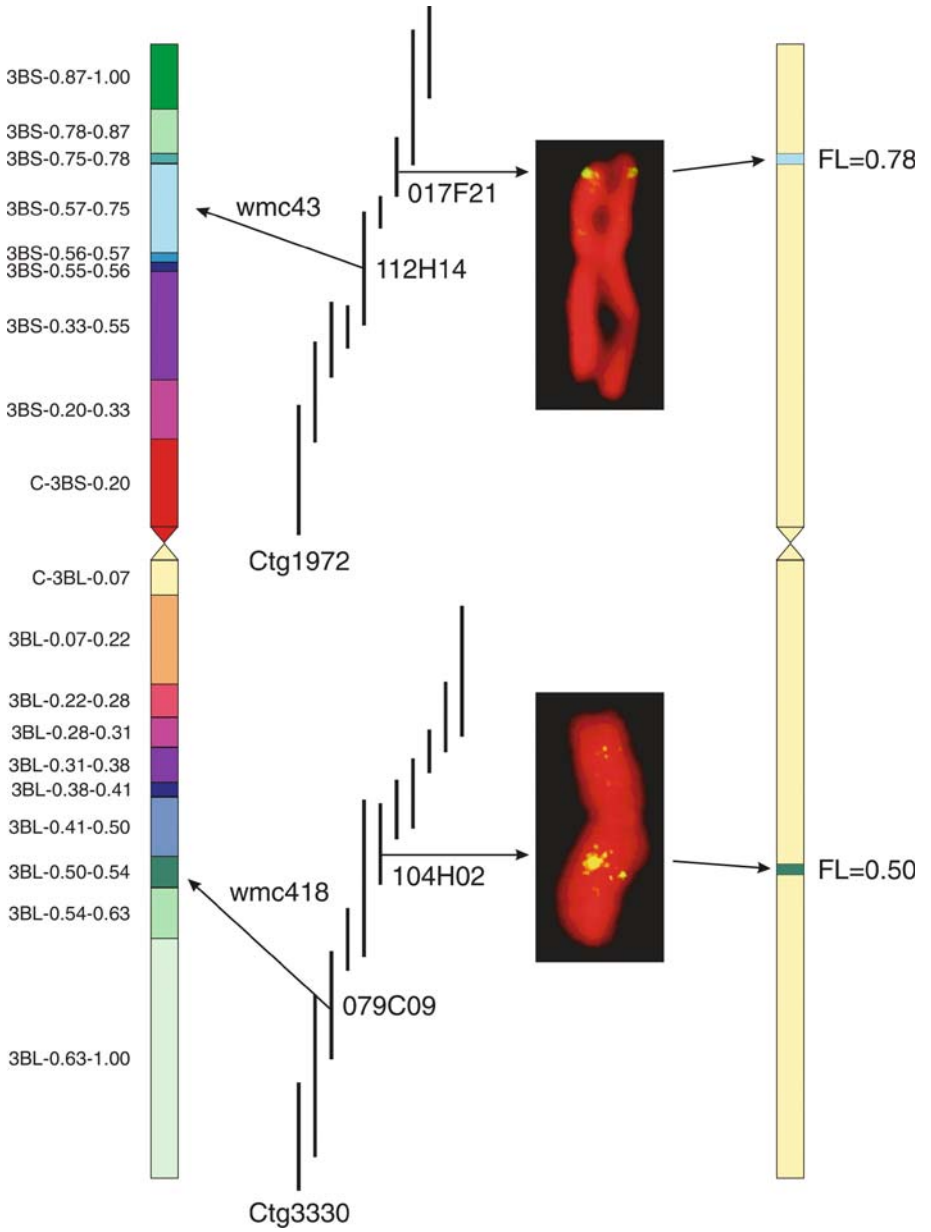

Figure 3. The integration of physical contigs and cytogenetic maps for wheat chromosome 3B. In the bin map of chromosome 3B (left), the bins are labelled by the fraction lengths (FL) of their borders (FL represents the percent of the chromosome arm length). Two BAC contigs (Ctg1972 and Ctg3330) were anchored to specific bins by means of the genetically mapped microsatellite markers wmc43 and wmc418. From each of the contigs a BAC clone containing low amounts of repetitive DNA was selected and localized by FISH to flow-sorted chromosome 3B. The cytogenetic map on the right shows the position of two BAC clones on chromosome 3B expressed as mean FL (average of 10 analyses). The analysis confirmed the position of the contigs on the chromosome and improved the precision of their location.

location T7HS·7HL-5HL, Fuchs *et al.* 1996), or from flow-sorted barley chromosome 1H (Cremer *et al.*, unpublished observation). This failure has been explained as being the result of a combination of the production of a weak signal from low-copy sequences and the dominance of dispersed repeats across the whole genome (Fuchs *et al.* 1996). Interestingly, similar arguments were used to explain the failure of biparameric flow karyotyping (Lucretti & Doležel 1997, Kovářová *et al.* 2007).

Isolated broad bean chromosomes have been shown to be suitable for the analysis of chromosome structure using scanning electron microscopy and immunostaining of chromosomal antigens (Schubert *et al.* 1993). This approach has been little explored in the cereals. However, a set of kinetochore proteins was successfully localized using immunofluorescent staining of isolated chromosomes of barley (ten Hoopen *et al.* 2000) in a study which also demonstrated the widespread conservation of SKP1 and CBF5 protein domains in plants. Immunostaining of sorted barley telochromosome variants of 7HS revealed that centromere-specific proteins and phosphorylated histone H3 were localized in the centromeric region even though the barley-specific satellite sequence was absent (Nasuda *et al.* 2005). This surprising result led to the conclusion that, for the assembly of the kinetochore, centromeric repeats are neither sufficient nor necessary.

Closing the circle ...

The parallel development of flow-sorting and BAC technology offers an exciting opportunity to develop specific probes for FISH and PRINS, and to apply these to sorted chromosomes. The high throughput and sensitivity of FISH on sorted chromosomes are ideal for its application as a cytogenetic mapping tool. The construction of physical maps is aided by prior localization of a BAC contig to a chromosomal segment, by orienting contigs with respect to the centromere and telomere, and by enabling the estimation of the size of gaps between adjacent contigs. This approach has been used to confirm the position of BAC contigs in wheat chromosome 3B (Bartoš *et al.*, unpublished observations, and Figure 3), while FISH has been applied to flow-sorted wheat and rye chromosomes to localize clones selected from chromosome-specific BAC libraries (Šafář *et al.* 2004, Janda *et al.* 2004, 2006). The initial success rate for the mapping of clones to a specific locus was low, due to interference from dispersed repeats, but this difficulty was largely overcome by the development of low-copy BAC subclones as FISH probes (Janda *et al.* 2006). The success of this approach relied on an ability to localize DNA sequences of about 2 kb to sorted chromosomes.

Future prospects

Practical methods for chromosome analysis and sorting to dissect the genomes of important cereals with complex genomes such as wheat, barley, rye and maize are now available. The possibility of constructing chromosome- and chromosome arm-specific BAC libraries holds out a promising prospect for the further development of genomics in these species, as they not only simplify the process of physical map construction and positional cloning, but also provide a viable strategy to sequence chromosomes one at a time. Moreover, the division of these large genomes into manageable portions helps to structure an international collaboration aiming to tackle large and polyploid genomes such as that of wheat. Thus, together with other applications of sorted chromosomes, flow cytogenetics now offers a versatile toolbox for the analysis of chromosome organization and function of large genome species. Along with the technical advantages of flow cytogenetics, it is important to keep in mind that the equipment cost and the operator technical skill level required are both high. For this reason it would be sensible to support the establishment of a small number of specialized laboratories capable of producing a volume of flow-sorted chromosomes and chromosome-specific genome resources sufficient to meet the requirements of downstream user laboratories. Once again therefore, flow cytogenetics may act as a stimulant for international collaboration.

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