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ORIGINAL PAPER

Isolated chromosomes as a new and efficient source of DArT markers for the saturation of genetic maps

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Abstract We describe how the diversity arrays technology (DArT) can be coupled with chromosome sorting to increase the density of genetic maps in specific genome regions. Chromosome 3B and the short arm of chromosome 1B (1BS) of wheat were isolated by flow cytometric sorting and used to develop chromosome- and chromosome arm-enriched genotyping arrays containing 2,688 3B clones and 384 1BS clones. Linkage analysis showed that 553 of the 711 polymorphic 3B-derived markers (78%) mapped to chromosome 3B, and 59 of the 68 polymorphic 1BS-derived markers (87%) mapped to chromosome 1BS, confirming the efficiency of the chromosome-sorting approach. To demonstrate the potential for saturation of genetic maps, we constructed a consensus map of chromosome 3B using 19 mapping populations, including some that were genotyped with the 3B-enriched array. The 3B-derived DArT markers doubled the number of genetic loci covered. The resulting consensus map, probably the densest genetic map of 3B available to this date, contains 939 markers (779 DArTs and 160 other markers) that segregate on 304 genetically distinct loci. Importantly, only 2,688 3B-derived clones (probes) had to be screened to obtain almost twice as many polymorphic 3B markers (510) as identified by screening approximately 70,000 whole genome-derived clones (269). Since an enriched DArT array can be developed from less than 5 ng of chromosomal DNA, a quantity which can be obtained within 1 h of sorting, this approach can be readily applied to any crop for which chromosome sorting is available.

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Introduction

During the past two decades, the development of an impressive range of DNA marker types (Doveri et al. 2008; Nguyen and Wu 2005) enabled significant advances in studying genetic diversity, following the transmission of loci of interest and facilitating marker-assisted selection and positional gene cloning. In the context of building physical maps, DNA markers are also essential for ordering BAC contigs and anchoring them to genetic maps (Meyers et al. 2004), an application that requires high marker densities and well-saturated genetic maps.

A large number of markers are especially important for crops with large genomes, such as barley, rye, and wheat. The hexaploid genome of bread wheat (*Triticum aestivum* L.)

is approximately 17-Gbp-long, and consists of three subgenomes: A, B, and D, each comprising seven chromosomes (Bennett and Smith 1976). When building the first physical map of a wheat chromosome, Paux et al. (2008) used 1,443 markers to anchor 680 contigs representing 75% of the chromosome 3B physical map. If this proportion holds for every chromosome, a minimum of 25,000 markers would be needed for a similar coverage of the entire wheat genome. Paux et al. (2008) also demonstrated that different classes of markers exhibited different distributions along chromosome 3B. Consequently, a large number of different types of markers are needed to ensure optimal coverage of entire genomes.

While markers are usually developed for entire genomes, an alternative option is to focus marker development on subgenomic regions. This approach should make it possible to generate high marker densities in regions of interest, such as a chromosome or a chromosome arm from which a gene is being cloned or for which a physical map is being constructed. Flow cytometric sorting and microdissection are the only methods suitable for isolation of DNA from selected chromosomes and chromosome arms (Doležel et al. 2004). However, the throughput of microdissection is low, and only flow cytometry can sort large quantities of chromosomes. To date, protocols for chromosome-sorting are available for 17 plant species, including some important legumes and cereals (Doležel et al. 2004). In bread wheat, flow cytometric chromosome analysis and sorting was reported by Vrána et al. (2000). The resulting distribution of chromosome DNA content (flow karyotypes) comprised four peaks. While the largest wheat chromosome (3B) formed a discrete peak and was readily sortable, the remaining 20 chromosomes clustered into three peaks. Subsequently, Kubaláková et al. (2002) demonstrated that with the exception of 3BL and 5BL, all wheat chromosome arms can be sorted as telosomes, which are maintained in stable cytogenetic stocks (Sears 1954). The 3BL and 5BL arms can be isolated from stocks carrying them as isochromosomes.

A number of applications of flow-sorted chromosomes have penetrated plant genetics and genomics during the last decade. These included physical mapping using PCR and FISH (Vláčilová et al. 2002; Valárik et al. 2004), the construction of chromosome and chromosome arm-specific BAC libraries (Šafář et al. 2004; Janda et al. 2004, 2006; Šimková et al. 2008a), the mapping of SNPs using oligonucleotide arrays (Šimková et al. 2008b), and shotgun-sequencing with the next generation sequencing technologies (Mayer et al. 2009). However, until now, DNA isolated from flow-sorted chromosomes has only been employed to develop SSR markers, and has not yet been combined with genotyping platforms with high multiplexing levels (Kofler et al. 2008; Román et al. 2004; Požárková et al. 2002).

Diversity arrays technology (DArT) markers are a relatively recent type of high-throughput marker system that excels in throughput and cost-effectiveness (Jaccoud et al. 2001; Wenzl et al. 2004; http://www.diversityarrays.com). DArT in its current implementation is a hybridization-based genotyping method that does not require sequence information and uses microarray technology to identify and type several thousands of dominant markers in parallel (Kilian et al. 2005). The technology has been established for more than 50 species and relies on DNA polymorphisms at (SNPs, InDels, methylation differences) or between (InDels) restriction-enzyme sites. DArT markers are widely used to analyze genetic diversity and develop genetic maps (Wenzl et al. 2004; White et al. 2008). Semagn et al. (2006) showed that DArT markers were distributed differently across the genome than SSR and AFLP markers, while Wenzl et al. (2004, 2006) and Akbari et al. (2006) reported preferential locations of DArT markers in gene-rich, subtelomeric regions in barley and wheat, respectively.

This study explores the possibility of developing DArT markers from specific regions of a plant genome by using DNA from flow-sorted chromosomes. We test this approach using the longest chromosome (3B) and one of the shortest chromosome arms (1BS) of wheat as pilots. We report not only on marker development but also on the saturation of the genetic map of chromosome 3B.

Materials and methods

Plant material

Seeds of five hexaploid wheat (*Triticum aestivum* L., $2n = 6x = 42$) cultivars 'Chinese Spring', 'Courtot', 'Renan', and 'Opata', and synthetic wheat W7984 (Van Deynze et al. 1995) were obtained from INRA (Clermont-Ferrand, France). These cultivars were chosen because they were used as parents of international reference mapping populations (Opata × W7984: ITMI population, Van Deynze et al. 1995; Chinese Spring × Renan: IWGSC population; Chinese Spring × Courtot population, Sourdille et al. 2003). Two ditelosomic lines of hexaploid wheat cv. 'Pavon 76' ($2n = 40 + 2t1BS$), carrying telocentric chromosomes 1BS of cultivars 'Begra' and 'Henika', were developed by centric misdivision of single chromosome substitution lines 1B of these cultivars into 'Pavon' by Prof. Adam Lukaszewski (University of California, Riverside, USA). The seeds were germinated in the dark at 25 ± 0.5°C on moistened filter paper for 3 days to obtain a root length of approximately 2–3 cm.

Chromosome sorting

Mitotic metaphase chromosomes were sorted using flow cytometry according to Vrána et al. (2000). To prepare liquid suspensions of intact chromosomes, roots of young seedlings were sequentially treated with hydroxyurea and amiprophos-methyl to accumulate meristematic root-tip cells at metaphase, and the synchronized root-tip meristems were fixed with formaldehyde (Vrána et al. 2000). Chromosomes were released by mechanical homogenization of 25 root tips in 1 mL ice-cold LB01 buffer (Doležel et al. 1989) and stained by 2 μg mL^{-1} DAPI (4′,6-diamidino-2-phenylindole). The samples were processed using a FACSVantage SE flow cytometer and sorter (Becton Dickinson, San José, USA). Batches of 10,000 chromosomes 3B and 35,000 telosomes 1BS were sorted into 50 μL deionized water in a PCR tube. The purity of sorted fractions was evaluated by FISH using probes for GAA microsatellite, *Afa* and telomeric repeat as described in Kubaláková et al. (2002).

DNA extraction

The DNA was prepared from flow-sorted chromosomes according to Šimková et al. (2008b). Briefly, the chromosomes were treated with proteinase K in a buffer consisting of 2.5 mM Tris (pH 8.0), 1.25 mM EDTA (pH 8.0), and 0.125% (w/v) SDS under gentle shaking at 50°C for 40 h. Two and 3 μg of proteinase K were added to the 10,000 and 35,000 chromosome samples, respectively. Another 1 or 1.5 μg was added after 20 h of treatment. The proteinase K and the buffer were subsequently removed using a Microcon YM-100 column (Millipore Corporation, Bedford, USA) in four rounds of centrifugation at 500×*g* for 14 min at 23°C. The amount of purified DNA was estimated using fluorometry.

Preparing DArT arrays containing chromosome-specific markers

Genomic representations were generated by digesting 1–2 ng of flow-sorted 1BS or 3B DNA with two units of both *Pst*I and *Taq*I (NEB, Beverly, USA). A *Pst*I adapter (5′-AC GAT GGA TCC AGT GCA-3′ annealed with 5′-CTG GAT CCA TCG TGC A-3′) was simultaneously ligated to *Pst*I ends using T4 DNA ligase (NEB). Approximately 5% of the ligation product was amplified in a 25-μL reaction using 5′-AT GGA TCC AGT GCA G-3′ as a primer. The amplification program was 94°C for 1 min, followed by 30 cycles of 94°C for 20 s, 58°C for 40 s, 72°C for 1 min, and 72°C for 7 min. Fragments from the resulting genomic representations were cloned into the *p*CR2.1-TOPO vector (Invitrogen, Mount Waverley, Australia) (Wenzl et al. 2004; Akbari et al. 2006).

Individual clones (384 clones derived from the two ditelosomic 1BS lines, 1,536 clones derived from chromosome 3B of 'Chinese Spring' and 1,152 3B-derived clones from the mixture of four other wheat cultivars; see Table 1) were grown in 384-well plates containing freezing medium consisting of 0.7 × LB medium supplemented with 4.4% glycerol, 8.21 g L^{-1} K_2HPO_4, 1.80 g L^{-1} KH_2PO_4, 0.50 g L^{-1} Na_3-citrate, 0.10 g L^{-1} $MgSO_4 \cdot 7H_2O$ and 0.90 g L^{-1} $(NH_4)_2SO_4$, 100 mg L^{-1} ampicillin, and 100 mg L^{-1} kanamycin. Inserts were amplified from 0.5 μL aliquots of bacterial cultures in 25 μL reactions containing 50 mM Tris, 6 mM HCl, 16 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM M13f primer (5′-GTT TTC CCA GTC ACG ACG TTG-3′), 0.2 μM M13r primer (5′-TGA GCG GAT AAC AAT TTC ACA CAG -3′), and a sufficient amount of Taq polymerase to obtain close-to-maximum yield. Amplification conditions were 95°C for 4 min, 57°C for 35 s, 72°C for 1 min, followed by 35 cycles of 94°C for 35 s, 52°C for 35 s, 72°C for 1 min, and 72°C for 7 min. Amplification reactions were dried at 37°C for 24 h, washed with 77% ethanol, dried at 37°C for 1 h, and dissolved in DArT-spotter buffer (50% DMSO, 1.5 M sorbitol, 0.1 M tri-ethanolamine–HCl, 0.5% (w/v) dextran, 0.02% (w/v) CHAPS).

The amplified inserts were grouped with other DArT markers derived from 234 whole-genome DNA samples of bread wheat, durum wheat and diploid, tetraploid and hexaploid wheat relatives, and printed in duplicate spots on SuperChip poly-L-lysine slides (Erie Microarray, Portsmouth, NH, USA) using a MicroGrid II arrayer (Biorobotics, Cambridge, UK). At least 24 h after printing,

Table 1 Purity of the chromosome fractions sorted from hexaploid wheat

Chromosome	Cultivar	Purity (%)[a]	
		Mean	SD
3B	Chinese Spring	88	1.58
3B	Courtot	92	2.08
3B	Opata	90	2.51
3B	Renan	93	1.53
3B	Synthetic	91	1.00
1BS	Begra	84	1.15
1BS	Henika	87	3.79

[a] Estimated by FISH independently on three microscope slides per cultivar. On each slide, 100 sorted particles were evaluated. The chromosomes were identified with probes for GAA microsatellite, *Afa* repeat family, and telomeric repeat

the slides were immersed in 92°C hot water for 2 min, transferred to a solution containing 100 μM DTT and 100 μM EDTA, and dried by centrifugation at 500×*g* for 7 min followed by incubation under vacuum for 30 min (Wenzl et al. 2004; Akbari et al. 2006).

DArT genotyping procedure

Approximately 20–100 ng of DNA samples of individual plants belonging to 19 different wheat mapping populations (with between 90 and 200 plants per mapping population) were processed as described above. Two replicate genomic representations were amplified from each sample, pooled, precipitated with one volume of iso-propanol, centrifuged at >3,000×*g* for 40 min at 30°C, washed with 77% ethanol, centrifuged at >3,000×*g* for 40 min at 30°C, and dried at room temperature. The representations were then labeled by adding 5 μL of labeling mix [10 mM Tris–Cl (pH 7.9), 10 mM $MgC1_2$, 50 mM NaC1, 1 mM DTT, 50 μM random decamers, 0.2 mM of dATP, dCTP, and dGTP, and 20 μM of dTTP], denaturing the solution at 95°C for 3 min, adding 5 μL of dye mix [10 mM Tris–Cl (pH 7.9), 10 mM $MgC1_2$, 50 mM NaC1, 1 mM DTT, 20 μM of either Cy3-dUTP or Cy5-dUTP (GE Healthcare, Rydalmere, Australia)], and 20 U μL^{-1} Klenow exo^- (NEB), and incubating the mixture at 37°C for 3 h (Wenzl et al. 2004; Akbari et al. 2006).

The resulting targets were added to 50 μL of a 500:50:2.5:2 mixture of ExpressHyb solution (Clonetech, Cheltenham, Australia), 10 mg mL^{-1} denatured herring-sperm DNA (Promega, Alexandria, Australia), 0.7 mg mL^{-1} FAM-labeled *p*CR2.1-TOPO polylinker (amplified with M13f and M13r primers), and 0.5 M EDTA (pH 8.0). The mixtures were denatured at 95°C for 3 min and hybridized to DArT arrays in a water bath at 62°C for 16 h. After hybridization, slides were washed in 1× SSC, 0.1% SDS (5 min), 1× SSC (5 min), 0.2× SSC (1 min), 0.02× SSC (30 s), and 100 μM DTT (30 s), and dried by centrifugation at 500×*g* for 7 min followed by incubation under vacuum for 30 min. The slides were scanned with a Tecan LS300 confocal laser scanner at 488, 543, and 633 nm (Tecan Austria, Grödig, Austria). Slide images were analyzed with DArTsoft (Diversity Arrays Technology P/L, Canberra, Australia) to identify polymorphic markers and call their allelic states (Wenzl et al. 2004; Akbari et al. 2006).

Constructing a consensus map for chromosome 3B

A total of 19 DH, RIL, and F2 wheat populations were genotyped for this study. All populations were genotyped with a set of whole genome-derived DArT markers which expanded from 3,456 to 6,528 over the period of approximately 2.5 years (polymorphic 1BS-derived markers were also added during this process). Four of these 19 populations were also genotyped with the 2,688 chromosome 3B-derived DArT markers, which were more recently generated using methods described above. The F2 population originates from a Chinese Spring × Renan cross, and is described in detail in Paux et al. (2008). The other maps were derived from DH and RIL populations genotyped at Triticarte P/L, with permission of the population owners to utilize the segregation data for mapping purposes. It is anticipated that the identity of all parents of these populations and possibly the segregation data as well will become available when consensus maps of all wheat chromosomes are generated and published.

Markers mapping to chromosome 3B were identified through linkage with other DArT markers for which chromosome assignments had previously been established. The marker order in each of the 20 chromosome-3B data sets (the F2 population data were split into a maternal and a paternal dataset) was established with EasyMap (Wenzl et al., in preparation), a program that implements the RECORD marker-ordering algorithm described by van Os et al. (2005). Poorly fitting markers (with more than 3% apparent double crossovers) were removed, and the marker order was re-optimized. Potential genotyping errors ($LOD_{error} > 4$; Lincoln and Lander 1992) were substituted with missing data, and the marker order was re-optimized again. The individual maps were then oriented and fed into PhenoMap software to construct a synthetic consensus map (GeneFlow Inc., Alexandria, USA). PhenoMap used the map with the largest number of markers to establish the order for all bridging markers it contains. It then added the remaining bridging markers in an iterative fashion by processing the remaining maps in descending order of the number of markers they have in common with the growing consensus map. Once all common markers were ordered, unique markers were added to the resulting map. To place a unique marker, its relative distance to the nearest flanking common markers on the component map was calculated and scaled to the equivalent distance on the consensus map.

Results

Chromosome sorting and preparation of DNA

Flow cytometric analysis of DAPI-stained chromosomes isolated from hexaploid wheat cv. 'Chinese Spring' results in histograms of relative fluorescence intensity (flow karyotypes) with four clearly resolved peaks (Fig. 1a). Three composite peaks (I, II, and III) contain various groups of chromosomes, while the rightmost peak is made of chromosome 3B, the largest wheat chromosome (Kubaláková et al. 2002).

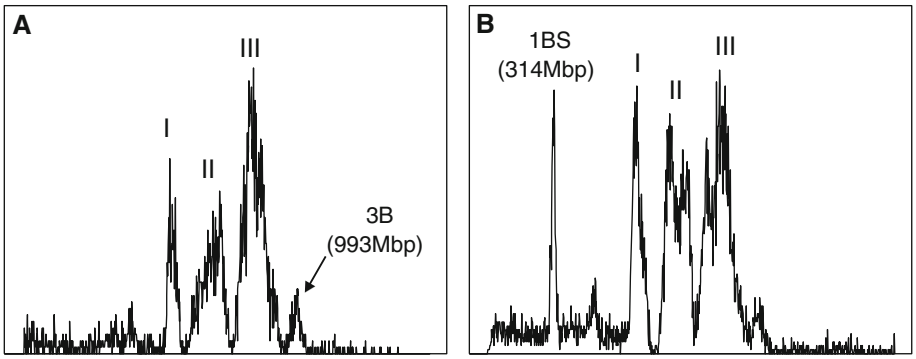

Fig. 1 Histograms of relative fluorescence intensities (flow karyotypes) obtained during the analysis of DAPI-stained mitotic chromosomes of bread wheat (*T. aestivum*). **a** Flow karyotype of cv. 'Chinese Spring' ($2n = 6x = 42$) consists of three composite peaks (I–III) representing groups of chromosomes and a peak representing chromosome 3B. Chromosomes were sorted from the 3B peak for DNA isolation. **b** Flow karyotype of a ditelosomic line of cv. 'Pavon 76' ($2n = 40 + 2t1BS$) carrying a telocentric chromosome 1BS of cv. 'Henika', which was sorted from the well-resolved peak. Molecular sizes of 3B and 1BS were determined considering 1C genome size 16,937 Mbp (Bennett and Smith 1976) and relative chromosome lengths as given by Gill et al. (1991). *X*-axis relative DAPI fluorescence intensity; *Y*-axis number of events

Similar flow karyotypes were observed after analyzing chromosome suspensions prepared from cultivars 'Courtot', 'Renan', 'W7984' and 'Opata' (data not shown) that were used for preparing the chromosome-specific DArT arrays in this study. The analysis of ditelosomic lines of cv. 'Pavon 76' carrying short arms of chromosome 1B (1BS) of cvs. 'Begra' and 'Henika' produced flow karyotypes that contained all four regular peaks (I–III, 3B) and an additional peak on the left of the composite peak I, representing chromosome 1BS (Fig. 1b).

Chromosome 3B and chromosome arm 1BS were sorted in batches of 10,000 and 35,000 copies, respectively. Sorting was performed at rates of 4–6 s^{-1} for 3B and 20 s^{-1} for 1BS, and one batch of chromosomes took approximately 45 min to sort. The average purity in the sorted fractions ranged from 88 to 93% for 3B and from 84 to 87% for 1BS, as determined by FISH on chromosomes sorted onto a microscopic slide using probes for GAA microsatellite, *Afa* and telomeric repeat (Table 1). The amounts of DNA obtained from individual batches of sorted chromosomes ranged from 6 to 15 ng per batch. This quantity was adequate as less than 5 ng of DNA is sufficient to prepare the genomic representations for the development of DArT markers.

Efficiency of chromosome-specific DArT marker development

To estimate the efficiency of DArT marker development from a specific chromosome (or chromosome arm), we identified genetic linkage between the 1BS- or 3B-derived markers and a set of several thousand DArT markers whose chromosomal locations had previously been established by analyzing the segregation data of approximately 100 wheat populations (Akbari et al. 2006; Triticarte P/L, unpublished data) (Fig. 2). Fifty-nine of the 68 polymorphic DArT markers (86.7%) derived from the 384 chromosome-1BS clones were assigned to chromosome 1B. Two of them were also mapped to a second locus on a different chromosome (5B and 7B). Another two markers (2.9%) showed linkage to homoeologous chromosomes 1A and 1D. Among the 711 polymorphic DArT markers derived from the 2,688 chromosome-3B clones, 553 (77.8%) were assigned to chromosome 3B. Four of them were multi-locus markers that reported a second locus on one of the two homoeologous chromosomes (3A, 3D). Twenty-five of the 711 polymorphic markers (3.5%) were exclusively assigned to homoeologous chromosomes 3A and 3D but not 3B.

A consensus map for chromosome 3B

Having confirmed the effectiveness of targeting marker discovery to specific chromosomes by using flow-sorted chromosomes, we next explored the degree of map saturation that could be achieved with this approach. We focused on chromosome 3B because of the greater number of markers developed for this chromosome. A consensus map of hexaploid wheat was built using the PhenoMap software to merge the order of markers present in 20 individual maps derived from 19 bi-parental populations (the F2-population dataset was split into two separate parental maps). The consensus map contains 939 markers, including 269 whole genome-derived DArT markers, 510 chromosome-3B-derived DArT markers, and 160 other markers (mostly SSR) (Fig. 3; ESM1). One whole genome-derived DArT marker (wPt-8718) was mapped to two separate loci within chromosome 3B, thus bringing the total number of loci on the consensus genetic map to 940.

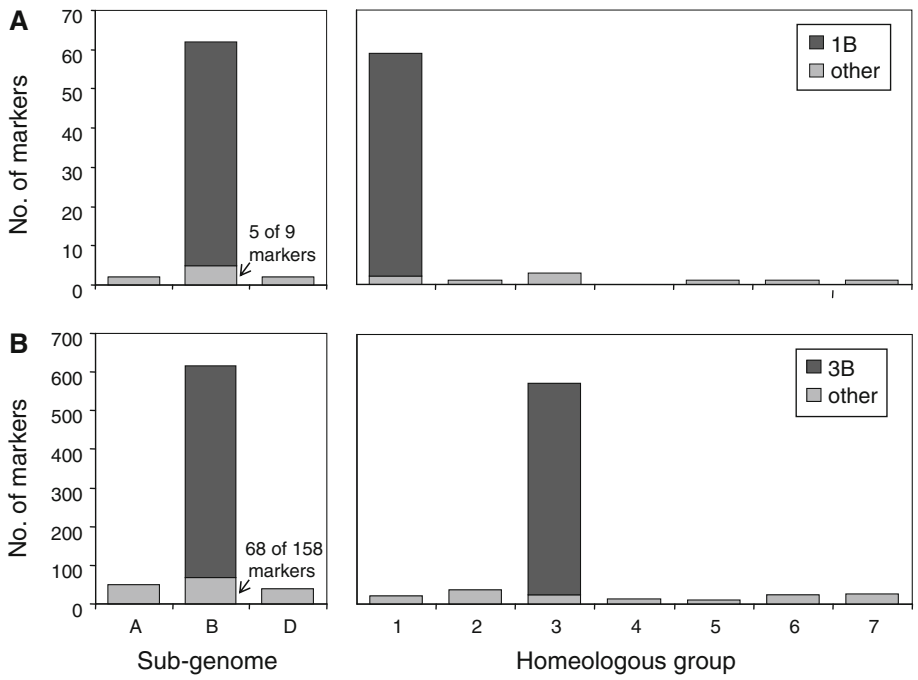

Fig. 2 Efficiency of targeting marker discovery to selected chromosomes. DArT markers cloned from genomic representations of flow-sorted 1BS chromosome arms (**a**) and 3B chromosomes (**b**) were mapped to chromosomes by comparing their segregation patterns in bi-parental mapping populations against those of a set of whole genome-derived DArT markers for which chromosome assignments had been established previously. *Black bars* denote markers assigned to the chromosomes from which markers had been derived. *Gray bars* represent markers assigned to other chromosomes. The small *inserts* in the two *left charts* indicate the number of markers mapping to non-target chromosomes on the B genome as a fraction to the total number of markers mapping to any of the non-target chromosomes

It is noteworthy that only 2,688 3B-derived clones (probes) needed to be screened to obtain almost twice as many polymorphic 3B markers (510) as identified previously by screening about 70,000 whole genome-derived clones (269).

Approximately one-third of the 940 loci on the consensus map (304) were genetically distinct (>0 cM distance between adjacent locus pairs at the resolution level of this study). DArT markers were mapped to 237 of these genetically distinct loci (77.7%) which was in accordance with their representativeness among the whole set of loci (83%). SSR markers mapped to 101 of these loci (33.4%). DArT markers cloned from whole-genome DNA preparations covered 142 loci, while those derived from flow-sorted 3B chromosomes covered 132 loci (Table 2).

To quantify more accurately the map-saturation improvement achieved by targeting marker discovery to flow-sorted 3B chromosomes, we removed from the consensus map all SSR markers and retained only those whole genome-derived DArT markers, which were polymorphic in the four populations used to map the 3B-derived markers. The number of genetically distinct loci more than doubled (from 92 to 192) when the 3B dataset is added to the whole-genome dataset (Table 2), despite the very limited genepool from which the 3B-specific markers had been developed (Table 1). This indicates that there is a great potential to further saturate the 3B consensus map by sorting chromosomes from a larger number of genetically diverse accessions.

Discussion

This study has explored the possibility of saturating sub-genomic regions of genetic maps of plant genomes by generating DArT markers from specific chromosomes and chromosome arms. The DNA which is needed to isolate such markers can be prepared from flow-sorting isolated chromosomes, a technique that has been applied to 17 plant species to this date (Doležel et al. 2004). We chose wheat with its complex, hexaploid genome to test this approach. Within wheat, we selected chromosomes with contrasting molecular sizes as test cases for this pilot study: chromosome 3B (993 Mbp), the longest chromosome of wheat, and chromosome arm 1BS, which is among the shortest chromosome arms with 314 Mbp (Doležel et al. 2009).

Chromosome sorting and preparation of DNA

Flow cytometric analysis of chromosomes stained by DAPI resulted in flow karyotypes, which corresponded to those obtained in earlier studies (Kubaláková et al. 2002).

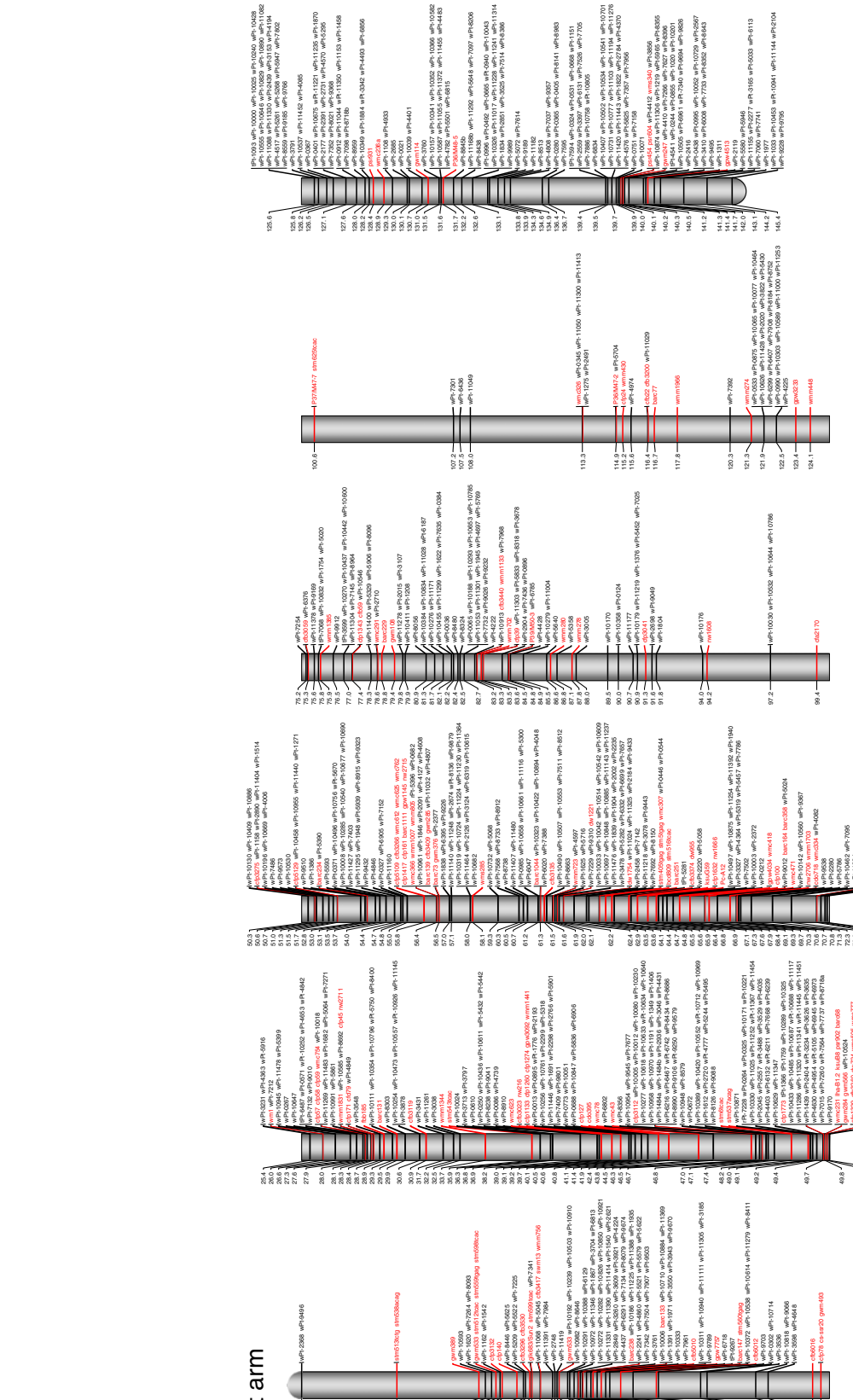

Fig. 3 Consensus map of chromosome 3B presented from *top* (short arm) to *bottom* (long arm), as derived from 20 individual maps built for 19 DH, RIL, and F2 populations. DArT markers are in *black*; SSR and other markers are labeled in *red* (see ESM1 for details)

Table 2 Genetically distinct loci on the wheat chromosome 3B consensus map as identified by different subsets of markers

Data set	Marker source	No. of individual maps in consensus map	No. of markers	No. of unique loci
DArT + SSR	Whole genome + 3B	20 (whole-genome DArTs), 4 (3B DArTs), 5 (SSR)	939[a]	304
DArT[b]	Whole genome	4	186[a]	92
	3B (flow-sorted)	4	510	132
	Whole genome + 3B	4	696[a]	192
	Whole genome	20	269[a]	142
	Whole genome + 3B	20 (whole genome), 4 (3B)	779[a]	237
SSR[b]	Whole genome	5	160	101

[a] One marker (wPt-8718) was mapped to two different loci at 49.7 and 127.6 cM

[b] DArT markers were developed in a parallel fashion by randomly drawing DNA fragments from genomic representations. They were not filtered for redundancy, for example by sequencing. In contrast, SSR markers were developed individually in a serial manner, a process during which redundancy is eliminated

This observation confirmed the reproducibility of flow karyotyping in hexaploid wheat. Flow-sorted chromosomes were identified by FISH with two different DNA probes, which turned out to be useful approach. While the use of GAA microsatellite and *Afa* as probes allowed the identification of chromosomes 3B and 1BS, in situ probing with a labeled probe for the telomeric repeat unambiguously identified intact 1BS telocentrics, thereby avoiding confusion of 1BS with chromosome arms of similar size generated by breakage of other chromosomes in the process of sample preparation and sorting (Kubaláková et al. 2002).

As less than 5 ng of DNA is sufficient to prepare genomic representations, there was no need for chromosomal DNA amplification. Direct use of isolated DNA to develop DArT markers eliminated any potential bias due to amplification, and preserved the DNA methylation status, which is important since the DArT protocol is based on the utilization of methylation-sensitive restriction enzymes. Thus, the requirement for non-amplified DNA excludes the use of chromosome microdissection in the DArT process, which in any case can only be applied to a small number of chromosomes (Hobza and Vyskot 2007). At the same time, a single batch of flow-sorted chromosomes (prepared within 1 h) is sufficient to selectively develop DArT markers from a chromosome and/or chromosome arm of interest.

Efficiency of chromosome-specific DArT marker development

Some markers derived from chromosome arm 1BS and chromosome 3B mapped to other genome regions, particularly to chromosomes belonging to the B genome (1BS: 5 of 9 markers or 55%; 3B: 68 of 158 markers or 43%). Several reasons may account for the fact that not all DArT markers developed from chromosomal DNA mapped to the source chromosomes. First, the purity of flow-sorted chromosome fractions only rarely reaches 100%, as these fractions are to some extent contaminated by other chromosomes (Doležel et al. 2007). For chromosome 3B, this B-genome preference could be explained by a slight contamination of the chromosome 3B fraction by other B-genome chromosomes, which constitute the majority of chromosomes in peak III next to the 3B peak (Kubaláková et al. 2002) (Fig. 1). The 1BS fraction could preferentially be contaminated with chromatids of chromosomes 1D, 4D, and 6D with relative fluorescence intensity similar to 1BS and/or short arms separated from intact chromosomes with molecular sizes similar to 1BS, such as 4AS, 6AS, 5BS, 2DS, 3DS, and 6DS. In fact, there is a striking similarity between the average purity of sorted 1BS fractions (85.5%) and the proportion of 1BS-derived markers mapping to chromosome 1B (86.7%). A larger difference was observed for chromosome 3B, where the average purity of the sorted fractions reached 90.8%, whereas 'only' 77.8% of 3B-derived markers mapped to chromosome 3B. The factors reducing the yield below the expected yield are unknown, but may be related to the size of chromosome 3B. Although most DArT markers reported a single locus, a minority of them reported an alternative locus on homoeologous chromosomes or repeated regions of the genome. The chance of cloning such markers should be greater for 3B than for other chromosomes, simply because with a size of 993 Mbp, 3B is the largest chromosome of wheat.

With 78–87% of markers developed from sorted chromosomes mapping to the original chromosomes, our strategy for targeted development of DArT markers appears quite efficient and holds a considerable promise to significantly increase the number of markers for a chromosome (or chromosome arm) of interest. The enrichment advantage is expected to be most pronounced in large genomes with many chromosomes such as wheat. With a molecular size of 314 Mbp, chromosome arm 1BS represents only 1.9% of the hexaploid wheat genome. With 86.7% of markers developed from the sorted arm being specific for 1B, the efficiency is 45-fold higher relative to

the whole genomic DNA approach. A 13-fold increase in efficiency was achieved for chromosome 3B, the largest wheat chromosome 3B (993 Mbp), which represents 5.8% of the genome.

A consensus map for chromosome 3B

As shown in Table 2, DArT markers cloned from whole-genome DNA preparations covered 142 loci, while those derived from flow-sorted 3B chromosomes covered 132 loci. The latter comparison is skewed against the 3B-derived markers for two reasons. First, the 3B-derived DArT markers were only genotyped in four populations, while the whole genome-derived DArT markers were assayed across 20 populations, thereby increasing their chance of being polymorphic in at least one of the parental combinations. Second, the 3B-derived markers were prepared from only five different wheat cultivars (see "Materials and Methods"; Table 1), while whole genome-derived markers were cloned from 234 different cultivars and accessions. The much narrower genetic diversity of accessions from which 3B-specific (and even more in case of 1BS-specific) markers were derived resulted in cloning fewer unique fragments corresponding to DArT alleles scored as "present" when compared to the "whole genome" approach, where we selected a broad range of genotypes to more broadly represent wheat genetic diversity. This lower number of unique cloned fragments limited the number of genetic loci that could be covered in this approach.

Conclusions

In this study, we demonstrate that the DArT platform can be coupled with chromosome sorting to target specific regions of a large genome to significantly increase the saturation of linkage maps of a chromosome of interest. Our pilot study on hexaploid wheat shows that DArT markers can be developed from DNA of sorted chromosomes at a 13–45-fold increased efficiency compared to DArTs developed from genomic DNA. More than 600 new markers were developed for wheat chromosome 3B and chromosome arm 1BS. The number of genetically distinct loci on the 3B consensus map was more than doubled.

Because each of the 42 chromosome arms can be isolated by flow-sorting (Kubaláková et al. 2002), any chromosome can now be saturated with a large number of DArT markers. The physical map of wheat is being constructed using a chromosome-by-chromosome approach, where individual laboratories develop maps for individual chromosomes (Feuillet and Eversole 2008). These laboratories can now develop saturated DArT maps for their specific chromosomes in an affordable and targeted manner.

In principle, this approach is applicable to any crop species for which methods of chromosome sorting are available (Doležel et al. 2004). Thus, DArT markers from flow-sorted chromosomes further increase the utility of the platform and provide a significant complement to other marker technologies in genetic, genomic, and breeding applications.

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