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Single-copy gene fluorescence *in situ* hybridization and genome analysis: *Acc-2* loci mark evolutionary chromosomal rearrangements in wheat

Tatiana V. Danilova, Bernd Friebe, Bikram S. Gill

Wheat Genetic and Genomic Resources Center, Department of Plant Pathology, Kansas State University, Manhattan, KS 66506

Abstract Fluorescent *in situ* hybridization (FISH) is a useful tool for physical mapping of chromosomes and studying evolutionary chromosome rearrangements. Here we report a robust method for single-copy gene FISH for wheat. FISH probes were developed from cDNA of cytosolic acetyl-CoA carboxylase gene (*Acc-2*) and mapped on chromosomes of bread wheat, *Triticum aestivum* L. (2n=6x=42, AABBDD), and related diploid and tetraploid species. Another nine full-length cDNA FISH probes were mapped and used to identify chromosomes of wheat species. The *Acc-2* probe was detected on the long arms of each of the homoeologous group-3 chromosomes (3A, 3B, and 3D), on 5DL and 4AL of bread wheat, and on homoeologous and nonhomoeologous chromosomes of other species. In the species tested, FISH detected more *Acc-2* gene or pseudogene sites than previously found by PCR and Southern hybridization analysis and showed presence/absence polymorphism of *Acc-2* sequences. FISH with the *Acc-2* probe revealed the 4A-5A translocation, shared by several related diploid and polyploid species and inherited from an ancestral A-genome species, and the *T. timopheevii* specific $4A^t$-$3A^t$ translocation.

Key words wheat, single-copy gene fluorescence *in situ* hybridization, acetyl-CoA carboxylase, karyotype evolution

Introduction

Bread wheat, *Triticum aestivum* L., is an allohexaploid (2n=6x=42, AABBDD) containing genomes of three species. Tetraploid wheats, 2n=4x=28, with AABB (Emmer group) and A^tA^tGG (Timopheevii group) genomes are the result of two separate hybridization events involving *T. urartu* Tumanian ex Gandilyan, the A-genome donor (Dvorak et al. 1993), and an outcrossing species closely related to the S genome of *Aegilops speltoides* Tausch, the ancestor of B and G genomes (Dvorak and Zhang 1990; Kilian et al. 2007). Hexaploid wheat arose from hybridization of cultivated emmer *T. turgidum* L. (AABB) (Nesbitt and Samuel 1996; Dvorak et al. 2004) and the D-genome donor *Ae. tauschii* Coss. (Kihara 1944; McFadden and Sears 1946; Dvorak et al. 1998).

The C- and N-banding karyotyping of bread wheat and its cultivated and wild relatives provided a foundation for analyzing the cytogenetic structure of the Triticeae (Gill et al. 1991; Friebe and Gill 1996). *In situ* hybridization with probes containing rye repeats (pSc119) were used to identify B-genome chromosomes (Rayburn and Gill 1985), and the Afa-family repeat from *Ae. tauschii* was used to identify the D-genome chromosomes (Rayburn and Gill 1987; Mukai et al. 1993). Fluorescence *in situ* hybridization (FISH) with probes containing a GAA-satellite sequence produced patterns similar to N-banding or C-banding on chromosomes of barley, rye, wheat, and *Aegilops* species (Gerlach 1977; Pedersen et al. 1996). Chromosome identification using repeated DNA probes is difficult because the abundance and distribution of repetitive elements can vary among homologous chromosomes within a species (Friebe and Gill 1994), or among chromosomes of closely related species (Badaeva et al. 1994; Dedkova et al. 2007).

A series of wheat aneuploid, deletion, and substitution lines have been used for gene mapping (Sears 1954; Endo and Gill 1996). The chromosome bin physical map of about 6,000 expressed sequence tags (ESTs) has been established using deletion stocks (Qi et al. 2004).

However, the deletion bins are large in size and loci within the bins cannot be ordered physically.

Using single-copy gene FISH has the potential of fine physical mapping and ordering of genes along the chromosomes, including chromosomal regions with low recombination rates. In wheat, gene-specific probes and probes for tandem repeats, including oligonucleotide probes, were used in indirect FISH with blocking to detect tandem repeats and gene clusters (Mukai et al. 1993; Pedersen and Langridge 1997; Turner et al. 1999; Li et al. 2003; Turnbull et al. 2003; Szakacs and Molnar-Lang 2007; Cuadrado et al. 2008 a, b). Indirect FISH uses nonfluorescent chemicals for labeling and needs an additional detection step for visualizing the hybridization site and amplifying of the signal. Single gene probes also were used in FISH with tyramide signal amplification (Perez et al. 2009). These methods need blocking, detection, and signal amplification steps and have not been used widely.

BAC clones were used as FISH probes for genic regions in species with small genomes and a low content of repetitive elements, such as *Arabidopsis* (Lysak et al. 2001), *Brachypodium* (Febrer et al. 2010; Ma et al. 2010), rice (Jiang et al. 1994), and sorghum (Woo et al. 1994; Kim et al. 2002). Bread wheat has one of the largest plant genomes (17 Gb), containing about 90% repetitive sequences, 70% of which are transposable elements and a large fraction of microsatellites and tandem repeats (Li et al. 2004). BAC-FISH in wheat painted either all chromosomes over their entire length, produced a genome-specific painting, or painted clusters of tandem repeats depending on the content of repetitive sequences in a BAC clone (Zhang et al. 2004 a, b).

For genomes enriched with repeats, a repeat-free probe for a particular genic region either can be produced from cDNA or developed from genomic DNA by sequence analysis and PCR amplification of the repeat-free region, and used in direct FISH as was shown in maize (Wang et al. 2006; Lamb et al. 2007; Danilova and Birchler 2008) and barley (Ma et al. 2010). In direct

FISH, fluorochromes are incorporated directly into DNA probes and the procedure does not need detection step.

As a model for probe development, we selected the wheat gene *Acc-2* encoding cytosolic acetyl-CoA carboxylase (ACCase). Plants have two forms of ACCase; one is present in plastids, catalyzing the *de novo* synthesis of fatty acids and another in the cytosol, involved in the synthesis of very long-chain fatty acids and secondary metabolites such as flavonoids and anthocyanins. Distinct from other species, where plastid ACCase is composed of four subunits some of which are encoded by chloroplast genes, in grasses both plastid and cytosolic forms of ACCase are encoded by nuclear genes, *Acc-1* and *Acc-2,* respectively (Sasaki and Nagano 2004). In wheat, the *Acc-1* genes were mapped on the short arms of the group-2 chromosomes (Gornicki et al. 1997) and *Acc-2* in the distal region of the long arm of the group-3 chromosomes and chromosome arm 5DL (Faris et al. 2001). A partly processed pseudogene *Ψ-Acc-2* with more than 90% identity to the *Acc-2* coding sequence was found in wheat, *T. urartu,* and *Ae. tauschii*. Six different sequences of the *Acc-2* gene and pseudogene are present in the wheat genome (Podkowinski et al. 1996; Huang et al. 2002); at least three of the *Acc-2* genes are expressed equally in young leaves (Podkowinski et al. 2003) and their coding sequences (6.3 kb) have 98% identity (Gornicki et al. 1994; Chalupska et al. 2008). The genes and pseodogenes are likely arranged in tandem repeats (Faris et al. 2001).

The objective of this research was to develop an *Acc-2*-specific probe and map *Acc-2* on chromosomes of wheat and its diploid and tetraploid progenitor species using multicolor direct FISH. For individual chromosome identification, probes for tandem repeats and microsatellites were used. For chromosomes lacking these markers, chromosome-specific full-length cDNA FISH markers were developed. The present work demonstrates the usefulness of direct FISH for physical mapping of genic sequences and studying chromosome rearrangements and will have broad applications in genome analysis of the Triticeae.

Materials and methods

Plant material

Seeds from the collection of Wheat Genetic and Genomic Resources Center, KSU, were used; *Triticum aestivum*, *T. urartu*, *T. monococcum*, *Ae. speltoides*, *Ae. tauschii*, *T. turgidum* subsp. *dicoccoides* (Körn. Ex Asch. & Graebn.) Thell., *T. turgidum* subsp. *dicoccum* (Schrank) Thell., *T. turgidum* subsp. *durum* (Desf.) Husn., and *T. timopheevii* (Zhuk.) Zhuk. (Table 1); double ditelosomic (dDt) and aneuploid ditelosomic (Dt) lines of cv. 'Chinese Spring': CSdDt1A (TA3132), CSdDt2A (TA3133), CSdDt3A (TA3134), CSdDt5A (TA3136), CSdDt6A (TA3137), CSdDt1D (TA3158), CSdDt2D (TA3146), CSdDt3D (TA3147-2), CSdDt4D (TA3148-2), CSdDt5D (TA3149), CSdDt6D (TA3150), CSdDt7D (TA3151), CSDt7DS (TA3130), CSDt7DL (TA3171) and cv. 'Canthatch': CTH Dt7DS (TA3068), CTH Dt7DL (TA3069).

Slide preparation and FISH procedure

Somatic chromosome preparations using the drop technique, direct probe labeling by nick translation, and the FISH procedure were as described previously (Kato et al. 2004; Kato et al. 2006) with minor modifications. In brief, 2-3 day-old root tips from germinated seeds (1.5-2.0 cm long) were cut and treated in a nitrous oxide gas chamber for 2 h 20 min, fixed on ice in cold 90% acetic acid for 10 min, washed and stored in 70% ethanol at −20°C. For slide preparation, roots were washed in tap water for 10 min and then in KCl buffer 5 min (75 mM KCl, 7.5 mM EDTA, pH 4); 3-5 meristems (1–2 mm long) were placed in 20 µl of 4% cellulase Onozuka R-10 (Yakult, Japan, Tokyo cat # 201069), 1% pectolyase Y23 (Karlan cat # 8006) in KCl buffer, and incubated for 55 min at 37°C. Digested meristems were washed for 5 min in ice-cold Tris–

EDTA buffer, pH 7.6, then three times in 100% ethanol. Meristems were dispersed by a needle in 15-25 µl of ice-cold acetic acid - methanol mix (9:1) and immediately dropped on to precleaned glass slides placed in a humid chamber. Dried preparations were UV cross-linked and used for hybridization on the same day. The mixture of probes and the slide preparation were denatured at 100°C separately. Oligonucleotide probes were not treated at 100°C unless they were used in a mixture with nick-translated probes. Probe amounts per slide are given in Table 2. The rest of the FISH procedure and washes were the same as in Kato et al. (2006). Chromosome preparations were mounted and counterstained with 4',6-diamidino-2-phenylindole solution (DAPI) or propidium iodide (PI) in Vectashield (Vector Laboratories, cat # H-1200, H-1300). Probe DNA was labeled with Fluorescein-12-dUTP (green) or Texas red-5-dCTP (red), (PerkinElmer, cat # NEL413001EA and NEL426001EA). Images were captured with a Zeiss Axioplan 2 microscope using a cooled charge-coupled device camera CoolSNAP HQ2 (Photometrics) and AxioVision 4.8 software (Zeiss). Images were processed using the Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA, USA).

Acc-2 FISH probes

Sequences of the *Acc-2* gene (U39321, chromosome 3AL) and cDNA (U10187, 3DL) were used to develop the probes. The gene sequence was analyzed with Repeatmasker software (Smit et al. 1996-2004), BLASTed against the Triticeae Repeat Sequence Database (http://wheat.pw.usda.gov/ITMI/Repeats, (Altschul et al. 1997)), and the repeat-free gene regions and the cDNA sequence were used for primer design (Table S1, Fig. 1).

Three week-old leaves of Chinese Spring were used for DNA and RNA extraction. DNA was isolated using the sodium bisulfite method (Schweizer et al. 1995). RNA was extracted with Trizol Reagent (Invitrogen, cat # 15596-026) according to the manufacturer's instructions. Selected *Acc-2* gene regions were amplified via PCR with genomic DNA as a template using

Sigma PCR reaction mix (Sigma, St. Louis, cat # P0982). For Reverse Transcriptase-PCR, cDNAs were amplified using RT–PCR system with Platinum Taq High Fidelity polymerase (Invitrogen, cat # 12574-030, 12574-035). PCR products of expected lengths were cut from 0.8 % agarose gel, purified with Qiagen Gel Extraction kit (cat. # 28706) and reamplified with the same primers. The purified DNA was labeled with Texas red by nick translation and used as a probe. All PCR products were tested separately by FISH and those that showed no background were cloned using pGEM-T vector (Promega, cat # A3600) and verified by sequencing. Plasmid inserts were amplified with standard primers (T7, Sp6), under standard conditions, purified with Invitrogene kit (cat # K-3100-01) and used for pooled FISH probe.

Chromosome identification using FISH to tandem repeats

Oligonucleotide probes (Table 2) were developed using wheat tandem repeat sequences available in public databases and synthesized by Integrated DNA Technologies with a flourochrome attached to the 5'-end. The GAA oligonucleotide probe produced a FISH pattern similar to that of N-banding (Gill et al. 1991) on all B-genome chromosomes, all A-genome chromosomes except 1A, and on three pairs of D-genome chromosomes of wheat (Fig. 2a). Sequences of the D-genome-specific Afa-family repeat pAs1 (Nagaki et al. 1995; Nagaki et al. 1998) were used to design several oligonucleotide probes. Two of them showed D-genome-specific labeling and were combined (Table 2). The FISH pattern of the pAs1 oligonucleotide probes was verified on a set of seven D-genome double ditelosomic lines (Fig. S1). The physically shorter arm of chromosome 7D is homeologous to the long arms of chromosomes 7A and 7B (Werner et al. 1992; Friebe et al. 1996). To verify the arm-specific GAA and pAs1 FISH patterns for chromosome 7D, ditelosomic Dt7DS and Dt7DL lines of Canthatch and Chinese Spring were used (Fig. S2). The D-genome karyotype is shown in Fig. 2b.

Additional oligonucleotide probes were developed for the 45S- and 5S rDNA-tandem repeats of bread wheat (Table 2, Fig. S3). For labeling the nucleolus organizing region (NOR) rRNA loci of wheat relatives, clone pTa71, containing a 9-kb insertion with 18S, 5.8S, and 26S rRNA wheat genes and intergenic spacers (Gerlach and Bedbrook 1979) was used as a probe.

Chromosome identification using full-length cDNAs

The wheat full-length (FL) cDNAs (Kawaura et al. 2009) used in this study were supplied by the National BioResource Project-Wheat, Japan (http://www.nbrp.jp). Long (>3 kb), FLcDNAs were selected from the Triticeae Full-Length cDNA database (TriFLDB) (Mochida et al. 2009). The chromosomal positions of selected cDNAs were detected by BLAST against expressed sequence tags (ESTs) mapped in the deletion bins of Chinese Spring (Qi et al. 2004; GrainGenes Database http://wheat.pw.usda.gov). FLcDNAs mapped on chromosomes of homoeologous groups 1, 3, 4, and 5 (Table 3) were selected, and the positions of the FLcDNA probes were verified by FISH on Chinese Spring chromosomes. Those probes that showed bright signals at the expected positions with no background were used to identify chromosomes of other species.

Results

Development of *Acc-2* FISH probe

The *Acc-2* gene should be an easily detectable FISH target because of its large size (transcribed part is 12.3 kb long, with 6.8 kb of coding sequence) (Chalupska et al. 2008). PCR primers were designed to produce a pooled FISH probe with a size of 5-6 kb (Table S1). Probes produced by PCR with genomic DNA as a template showed background hybridization to all chromosomes (Fig. S4) except for probe 3g, which did not include any introns. Apparently, despite sequence

analysis, some repetitive elements were not detected in *Acc-2* introns because of incompleteness in the wheat repeat databases. PCR products 1c, 2c, and 3c (Table 2, Fig. 1) produced by RT-PCR showed no background when used as FISH probes. They were cloned, verified by sequencing, and showed 97-100 % similarity to the *Acc-2* cDNA U10187. The pooled probe with a total length of 5,970 bp, containing the mixture of 1c, 2c and 3c, PCR products, detected *Acc-2* loci on the long arms of chromosomes 3A, 3B, 3D, 4A and 5D of Chinese Spring (Fig. 2c), Wichita, and TAM107. No FISH signals were detected on the short arms of the group-2 chromosomes (Fig. S5), where the *Acc-1* gene was mapped (Gornicki et al. 1997).

Full-length cDNA FISH probes

Some chromosomes of diploid wheat and *T. timopheevii* with *Acc-2* signals could not be distinguished based on their morphology, NOR site, or GAA pattern. To identify these chromosomes, FLcDNAs were selected from the TriFLDB database by sequence similarity to mapped ESTs. Each of the FLcDNA FISH probes hybridized to the expected positions on the three homoeologous chromosomes of the A, B and D genomes of Chinese Spring. An exception was FLcDNA 5S-1 (tplb0027f03), which showed 98% similarity to the sequence of EST mapped on group-3 chromosomes but hybridized to the short arms of chromosomes of group-5 (Table 3). Because FLcDNA clones were checked only by PCR to verify the size of the inserts but were not verified by sequencing, the FLcDNA 5S-1 clone may contain a wrong insert. In total, we produced nine FLcDNA chromosome-specific FISH markers; one or two markers for each arm of group-1, -4 and -5 chromosomes and one marker for the short arm of group-3 chromosomes. The size of the FLcDNA FISH probes ranged from 1,491 bp to 5,094 bp (Table 3).

Development of FISH karyotypes and mapping of *Acc-2* genes on chromosomes of wheat and *Aegilops* species

For developing FISH karyotypes, probes pTa71 and the GAA- or pAs1-oligonucleotide were used and the patterns and morphologies were compared with existing C- or N- banding karyotypes. To map *Acc-2*, the pooled *Acc-2* probe was hybridized together with GAA- or pAs1-oligonucleotide probes. If it was not possible to identify chromosomes using the repeats, the oligonucleotide probes or the *Acc-2* probe were combined with chromosome-specific FLcDNA probes in multicolor FISH.

A-genome diploids Although the morphology and C-banding pattern of chromosomes of *T. monococcum* and *T. urartu* were reported to be similar to those of the A-genome chromosomes of *T. turgidum* and *T. aestivum* (Friebe and Gill, 1996), their GAA banding was different in the accessions analyzed. All A-genome chromosomes of bread wheat have specific GAA signals except chromosome 1A (Fig. 2a). In *T. urartu,* we found two chromosome pairs with some minor GAA signals, one chromosome pair with the NOR signal and one with both, the NOR and a bright GAA signal (Fig. 3a). However it was not possible to determine their homeology unambiguously. In *T. monococcum*, only chromosome 4A can be distinguished by a bright GAA signal near the centromere (Friebe and Gill 1996). Minor GAA signals were detected on chromosomes which might be either 2A or 3A and on NOR chromosomes 1A or 5A (Fig. 3c).

We observed three chromosome pairs with *Acc-2* FISH sites in both *T. urartu* and *T. monococcum*. In *T. urartu*, *Acc-2* signals were detected on the long arm of a chromosome with the NOR site and bright GAA signal on the short arm near the centromere, which could be either chromosome 1A or 5A; on a small metacentric chromosome with no GAA signals, which could be either 4A or 6A; and on a larger submetacentric chromosome with a minor GAA signal on the short arm, which could be either 2A or 3A based on size, arm ratio, and the absence of the NOR site (Fig. 3a, b). Using FLcDNA probes, the chromosomes with *Acc-2* loci were identified as 1A, 3A and 4A (Fig. 4a).

In *T. monococcum*, the *Acc-2* FISH sites were detected on chromosome 4A with the bright proximal GAA signal. Two other chromosome pairs with *Acc-2* foci were identified with FLcDNA FISH markers as 1A, and 3A. (Fig. 3c, d; Fig. 4b). Thus, both diploid *Triticum* species have *Acc-2* FISH loci on homologous chromosome arms 1AL, 3AL, and 4AL.

***Ae. speltoides* (SS)** Two accessions of *Ae. speltoides* were analyzed by FISH. The GAA-FISH pattern identified all seven pairs of chromosomes. The accession TA2368 had five *Acc-2* FISH sites: four on the long arms of chromosomes 3S and 5S and one on the long arm of one chromosome 1S (Fig. 3e, f). Another accession, TA2780, had four *Acc-2* FISH sites on the long arms of chromosome pairs 3S and 5S.

***Ae. tauschii* (DD)** The chromosomes of *Ae. tauschii* were identified using the FISH karyotype of the D genome of bread wheat. However, chromosome 5D differed from 5D of wheat by the presence of the NOR. *Acc-2* loci were detected on *Ae. tauschii* chromosome arms 3DL and 5DL (Fig. 3g, h).

AB-genome tetraploids The chromosomes of both wild and cultivated tetraploid accessions were identified by their GAA-FISH patterns, which are similar to those of the A- and B-chromosomes of *T. aestivum*. We tested four *T. turgidum* subsp. *dicoccoides* accessions of different geographic origins (Table 1). All were polymorphic in the number of *Acc-2* FISH sites and their distribution. In the accession TA73 (Lebanon) and TA1392 (Israel), five *Acc-2* loci were detected, two in the A-genome chromosome arms 3AL and 4AL and three on the B-genome chromosome arms 1BL, 3BL, and 5BL (Fig. 3 i, j). In the accessions TA1385 (Iraq) and TA84 (Turkey), four *Acc-2* loci were detected on chromosome arms 3AL, 4AL, 3BL, and 5BL.

In *T. turgidum* subsp. *dicoccum,* four *Acc-2* loci were detected on chromosome arms 3AL, 4AL, 1BL, and 3BL. In three cultivars of *T. turgidum* subsp. *durum,* we detected three *Acc-2* loci on chromosome arms 3AL, 4AL, and 3BL (Fig. 3k, Table 1).

***T. timopheevii* (A^tA^tGG)** Some chromosomes of A^t genome of *T. timopheevii* can be distinguished by their GAA-FISH patterns (2A, 7A), NOR site (6A), and by their size and arm

ratios (1A). G-genome chromosomes have specific GAA-FISH pattern, differ in size and arm ratio, and can be distinguished using a generalized C-banding karyotype of *T. timopheevii* subsp. *araraticum* Jakubz., a wild relative of *T. timopheevii* (Badaeva et al. 1994). Five chromosome pairs, two belonging to the A^t genome and three to the G genome, had *Acc-2* loci on their long arms. Chromosome $1A^t$ is the smallest in the A^t genome and was identified on this basis. The second, a submetacentric chromosome with two clearly distinguishable *Acc-2* FISH signals, could be either $3A^t$, $4A^t$, or $5A^t$. The G-genome chromosomes with *Acc-2* signals were similar to 1G, 3G, and 5G of *T. timopheevii* subsp. *araraticum*. FLcDNA FISH probes were used to identify these chromosomes, although, the *T. timopheevii* specific rearrangements can hamper this approach. Translocations T1GS/6A^tS, T1GS/4GS, T4GS/4A^tL and T4A^tL/3A^tL were shown to be present in distinct wild populations and cultivated *T. timopheevii* (Gill and Chen 1987; Jiang and Gill 1994; Maestra and Naranjo 1999; Rodriguez et al. 2000). Unlike *Acc-2*, the FLcDNA FISH probes used in our experiment were mapped in regions that are not involved in these translocations (Table 3), thus, allowing unambiguous identification of *T. timopheevii* chromosomes. The chromosome arms with *Acc-2* FISH signals were identified as 1A^tL, 4A^tL (two distinct *Acc-2* loci), 1GL, 3GL, and 5GL (Fig. 3 l, m, Fig. 4c).

In some chromosomal spreads of different species, it was possible to distinguish two closely located *Acc-2* FISH signals: on chromosome arms 3AL and 3DL of Chinese Spring, 4AL of Wichita, 3BL and 3DL of TAM107, 3AL of *T. urartu* and 3DL of *Ae. tauschii* (Fig. S6, Table 1).

Discussion

Single-copy gene FISH and oligonucleotide probes for wheat genome analysis

Here we report a fast and reliable single-copy gene FISH technique for wheat using nitrous oxide gas treatment for arresting cells at metaphase, the drop technique for somatic chromosome preparation, and direct probe labeling by nick translation (Kato et al. 2004; Kato et al. 2006). This procedure does not need detection and blocking steps, which were previously being used in indirect FISH to visualize gene clusters and repeats (Mukai et al. 1993; Pedersen and Langridge 1997; Turner et al. 1999; Li et al. 2003; Turnbull et al. 2003; Szakacs and Molnar-Lang 2007; Cuadrado et al. 2008a, b) or a signal-amplification step in tyramide-FISH (Perez et al. 2009). The direct FISH technique is sensitive enough to detect cDNA probes with a size of 3 kb on wheat somatic chromosomes (Fig. 4, Table 3). Cheap and reliable oligonucleotide FISH probes can be used for chromosome identification and detecting large-scale chromosomal rearrangements in the Triticeae or can be combined with single-copy gene probes in multicolor direct FISH for studying small-scale rearrangements and fine mapping.

FISH mapping of *Acc-2* genes

The *Acc-2* gene was mapped by Southern hybridization analysis of wheat aneuploid and deletion stocks on chromosomes 3A, 3B, 3D, and 5D of *T. aestivum* and by linkage mapping on chromosomes 3DL and 5DL of *Ae. tauschii* (Faris et al. 2001). In TAM107, six copies of the *Acc-2* gene or pseudogene sequences were found by PCR cloning and DNA sequence analysis. Two copies of the gene and one pseudogene were found in diploid *T. urartu* and *T. monococcum*; two copies of the gene were found in *Ae. speltoides,* and one gene and one pseudogene were found in *Ae. tauschii*. In the tetraploid species *T. turgidum* subsp. *dicoccoides* and *T. timopheevii,* three copies of the *Acc-2* gene or pseudogene sequences were isolated (Faris et al. 2001; Huang et al. 2002), (Table 1). We applied FISH mapping to the same accessions of wheat and *Aegilops* species, except for *T. urartu* and some additional accessions.

In the present study, *Acc-2* cDNA was used as a FISH probe. The sequences of *Acc-1* and *Acc-2* cDNAs (U10187 and AF029895) are approximately 73% similar when compared by NCBI BLASTN (Altschul et al. 1997). Three *Acc-1* loci were mapped on the short arms of the group-2 chromosomes and on 2DS of *Ae. tauschii* (Gornicki et al. 1997). The stringency of FISH with the *Acc-2* cDNA pooled probe based on the GC content of the probe, the hybridization and washing temperatures, and buffer composition is 65-70% (Schwarzacher and Heslop-Harrison 2000). We did not observe any FISH signals on group-2 chromosomes of bread wheat or *Ae. tauschii*; hence, our hybridization stringency is higher than 73% and sensitive enough to distinguish between the *Acc-1* and *Acc-2* sequences.

FISH revealed *Acc-2* loci on chromosome arm 4AL of bread wheat in three cultivars with distinct pedigrees and additional copies of *Acc-2* genes or pseudogenes were found in other genomes: three versus two in *Ae. speltoides,* and five versus three in *T. turgidum* subsp. *dicoccoides* and *T. timopheevii* (Table 1). The FISH resolution on condensed mitotic chromosomes is not very high. The axial resolution of FISH on maize somatic chromosome is 3-8 Mb, but it is possible to distinguish separate FISH signals that are about 200 kb apart (Danilova and Birchler 2008). Double *Acc-2* FISH signals were observed on chromosomes 3A, 3D, 3B, and 4A of bread wheat; $4A^t$ and 3G of *T. timopheevii;* and 3D of *Ae. tauschii* (Fig. S6), indicating that on some chromosomes, *Acc-2* genes or pseudogenes are arranged in tandem repeats, which agrees with previous studies (Faris et al. 2001; Huang et al. 2002). Thus, the total number of undetected *Acc-2* gene or pseudogene copies may even be higher. In polyploid species, the PCR cloning approach and Southern hybridization analysis may not detect copies of homoeologous or orthologous genes because of sequence divergence or high sequence identity (Feuillet et al. 2001; Huang et al. 2002; Caldwell et al. 2004). FISH can help in mapping and evaluating the copy number of these genes.

Change of *Acc-2* chromosome location and copy number during Triticeae evolution

Genomes of newly formed allopolyploids are prone to rapid changes leading to cytological, genetic and epigenetic diploidization through elimination of DNA sequences, gene silencing or changing functions of homeologous genes (Dvorak et al. 2004; Pumphrey et al. 2009). Locus deletions followed polyploidization event happen more frequently at the distal ends of wheat chromosomes with high recombination rate (Dvorak et al. 2004). Some synteny perturbations between wheat homoeologous chromosomes originated at the diploid level. However, the homoeologous genomes of young allopolyploid species like *T. aestivum* are still largely collinear i.e. composed of homoeologous genes at equivalent positions along the chromosomes (Feuillet et al. 2001; Akhunov et al. 2003).

We observed that the number of *Acc-2* loci varied from two to five and some consisted of tandemly organized, duplicated copies (Table 1). We detected the *Acc-2* FISH signals on the long arm of group- 1, 3, and 5 chromosomes of S-genome of *Ae. speltoides* (TA2368); the B genome of *T. turgidum* subsp. *dicoccoides* and the G genome of *T. timopheevii*; on the long arms of chromosome 1A and 3A of *T. urartu* and *T. monococcum*; $1A^tL$ of *T. timopheevii;* and on the 3DL and 5DL of *Ae. tauschii* and *T. aestivum*. In barley, two copies of *Acc-2* gene were found (Faris et al. 2001). By comparing the wheat *Acc-1* and *Acc-2* cDNA sequences (AF029895 and U10187) against the genome sequence of barley (VIROBLAST (Deng et al. 2007), http://webblast.ipk-gatersleben.de/barley/viroblast.php), we found one sequence with 95% similarity to wheat *Acc-1* on barley chromosome arm 2HS and two sequences with 96% and 90% similarity to wheat *Acc-2* on chromosomes arms 3HL and 5HL, respectively. Barley chromosomes are highly collinear to the corresponding wheat chromosomes (Dubcovsky et al. 1996). The comparison of the wheat *Acc-1* and *Acc-2* sequence with the rice genome (NCBI BLASTN (Zhang et al. 2000)) detected a sequence similar to *Acc-1* on the long arm of rice chromosome 5 and to *Acc-2* on the long arm of rice chromosome 10. Both rice chromosomes are homologous to the group-1 chromosomes of wheat (Sorrells et al. 2003). Based on these data and

our FISH mapping results, we assume that the original location of multicopy *Acc-2* genes in the diploid progenitor of Triticeae was on the long arm of chromosomes 1, 3, and 5 (Fig. 5).

4AL/5AL translocation In the A genome of all diploid and polyploid species tested in this study, *Acc-2* loci were detected on chromosome arm 4AL but not on 5AL. Chromosome 4A of *T. monococcum*, *T. turgidum,* and bread wheat is known to have a rearranged structure that resulted from a 4AL/5AL translocation, which occurred at the diploid level. Further rearrangements involving chromosome arms 7BS, a paracentric and two pericentric inversions, took place at the tetraploid level (Naranjo et al. 1987; Devos et al. 1995; Mickelson-Young et al. 1995; Miftahudin et al. 2004). Our results confirm the presence of the A-genome-specific translocation T4AL/5AL that is shared by several related species and is also present in the derived A genomes of allopolyploid species (Jiang and Gill 1994; Rodriguez et al. 2000) (Fig. 5).

3A^{t}L/4A^{t}L translocation *Acc-2* FISH signals in *T. timopheevii* were observed on chromosome arms 1A^{t}L and 4A^{t}L, similar to the A-genome progenitor species but were absent on chromosome arm 3A^{t}L and duplicated on 4A^{t}L. According to Maestra and Naranjo (1999) and Rodriguez et al. (2000), one of the *T. timopheevii* species-specific translocations involved chromosome arms 4A^{t}L and 3A^{t}L. Therefore, of the two distal *Acc-2* loci detected on the chromosome arm 4A^{t}L, the proximal FISH site marks the 5AL segment and the distal signal marks the 3AL segment. This Timopheevii lineage-specific chromosome can be described as T4A^{t}S·4A^{t}L-5A^{t}L-3A^{t}L. The presence of a 3A^{t}L segment on T4A^{t}S·4A^{t}L-5A^{t}L-3A^{t}L is also indicated by the high metaphase I pairing of this segment with the 3AL arm of *T. turgidum* (Rodriguez et al. 2000).

It is likely that as a result of a reciprocal translocation the distal 5A^{t}L segment of T4A^{t}S·4A^{t}L-5A^{t}L was translocated to the distal end of the 3A^{t}L arm, and chromosome 3A^t can be described as T3A^{t}S·3A^{t}L-5A^{t}L. Two lines of evidence support this hypothesis. Rodriguez et al. (2000) observed very low pairing between 3AL of *T. turgidum* with 3A^{t}L of *T. timopheevii*. It

has been shown previously that non-homology at the distal ends leads to a drastic reduction in meiotic metaphase I pairing (Curtis and Lukaszewski, 1991; Gill and Friebe, 1998). In addition, Gill and Chen (1987) observed that chromosome $4A^m$ (T4AS·4AL-5AL) of *T. monococcum* paired in the form of a quadrivalent with chromosome 3A (3AS·3AL) of *T. turgidum* and chromosomes $3A^t$ ($T3A^tS·3A^tL$-$5A^tL$) and $4A^t$ ($T4A^tS·4A^tL$-$5A^tL$-$3A^tL$) of *T. timopheevii* in 23.3% of the pollen mother cells analyzed. This configuration is only possible if $3A^t$ is, in fact, a translocation chromosome $T3A^tS·3A^tL$-$5A^tL$.

S, B, G genome polymorphism *Ae. speltoides*, the putative donor of the B and G genomes in AABB and A^tA^tGG tetraploids, is a cross-pollinating species and has the highest level of diversity compared with other diploid species (Kilian et al. 2007). The S genome of *Ae. speltoides* and B/G genomes of tetraploid species were polymorphic for the *Acc-2* FISH pattern. The *Ae. speltoides* TA2368 (Turkey) had five *Acc-2* FISH signals, four on the 3SL and 5SL arms and one on the chromosome arm 1SL. *Ae. speltoides* TA2780 (Israel) had *Acc-2* loci on chromosome arms 3SL and 5SL. In the B and G genomes of wild *T. turgidum* subsp. *dicoccoides,* TA73 (Lebanon), TA1392 (Israel), and *T. timopheevii*, *Acc-2* loci were detected on three chromosome pairs of group 1, 3, and 5. In two other *T. turgidum* subsp. *dicoccoides* accessions, from Iraq and Turkey, *Acc-2* loci were present on 3BL and 5BL. In cultivated *T. turgidum* subsp. *dicoccum* TA10492 (Ethiopia), the *Acc-2* FISH signals were detected on 1BL and 3BL. It is interesting to note that the *T. turgidum* subsp. *dicoccoides* accessions with similar *Acc-2* distribution have similar geographic origin, either western (Israel, Lebanon) or central-eastern (Iraq, Turkey). The origin of the Emmer wheat in Ethiopia is unknown. The wild Emmer from the central-eastern group is closer to cultivated populations (reviewed in Nesbitt and Samuel 1996; Haudry et al. 2007). In three cultivars of domesticated *T. turgidum* subsp. *durum* and *T. aestivum*, the *Acc-2* FISH signal was detected only on chromosome arm 3BL (Table 1). The ancestors of *T. turgidum* and *T. timopheevii* originated as a result of independent polyploidization events involving *Ae. speltoides* as a source of the G and B genomes and

T. urartu as a source of the A genome (Jiang and Gill 1994; Rodriguez et al. 2000; Kilian et al. 2007). Hexaploid wheat has no wild form; it originated as a hybrid of cultivated *T. turgidum* and *Ae. tauschii* (McFadden and Sears 1946; Dvorak et al. 2004; reviewed in Matsuoka 2011). Whether or not chromosomes 1BL and 5BL of *T. turgidum* were involved in rearrangements that resulted in the loss of *Acc-2* loci in wild and domesticated Emmer or different forms of *Ae. speltoides* were involved in several hybridization events in western and central eastern regions resulting in polymorphic AABB tetraploids is unknown.

Conclusions

1. Oligonucleotide probes were found to be useful for identifying chromosomes of bread wheat and related tetraploid and diploid species except the A genomes of *T. urartu*, *T. monococcum,* and *T. timopheevii*, which do not have enough tandemly organized GAA and pAs1 repeats detectable by direct FISH.

2. Single-gene probes were developed from wheat cDNAs and used for identifying chromosome arms and mapping genes by direct FISH. Currently, we are developing a set of single-gene FISH markers that will cover all wheat chromosome arms.

3. The PCR approach, with genome-specific primers, or Southern hybridization analysis sometimes may not reveal all copies of genes or pseudogenes in polyploid species. FISH with *Acc-2* probe revealed the presence of additional *Acc-2* copies in A, B, D, and G genomes in the Triticeae.

4. The distribution of *Acc-2* loci, representing a multigene family on chromosomes of A, B, G, S, and D genomes of the analyzed species was variable, showed that the collinearity of the genomes is not perfect. On the other hand, genes present as a single locus in each genome can be a reliable chromosome landmark, as was shown for nine FLcDNAs.

5. FISH with the *Acc-2* probe revealed the presence of chromosome rearrangements that originated at the diploid or tetraploid level and are shared by several related species. The A-

genome specific 4AL-5AL translocation was detected in diploid and polyploid species including both the Emmer and Timopheevii groups, and the $4A^tL$-$3A^tL$ translocation was detected in *T. timopheevii*. Thus, single-copy gene FISH, along with other methods, can be a useful tool in phylogenetic and evolutionary studies.

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Table 1 *Acc-2* mapping and detecting copy number: comparison of FISH and results of Southern analysis of wheat aneuploidy stocks and genomic DNA sequence analysis (from Faris et al. 2001; Huang et al. 2002)

Species, genome	Accession (origin)	Number of chromosomes with *Acc-2* FISH signal	Chromosomes with *Acc-2* FISH signals (chromosome specific cytological marker is described)	Position on chromosome and copy number (Faris et al., 2001; Huang et al., 2002)	C-banded karyotypes used for chromosomes identification
T. aestivum AABBDD	Chinese Spring	10	**3AL** (two copies), **4AL**; **3BL;** **3DL, 5DL**	**3AL, 3BL, 3DL, 5DL**	(Gill et al., 1991; Friebe et al. 1996)
	Wichita (USA)	10	The same as Chinese Spring; 4AL – 2 *Acc-2* copies*	6 sequences	
	TAM107 (USA)	10	The same as Chinese Spring; 3BL – 2 *Acc-2* copies*		
T. urartu AA	TA766	6	**1AL** (NOR, FLcDNAs 1S-1, 1L-2, co-localized with GAA on 1S), **3AL** (FLcDNA 3S-2, co-localized with GAA), **4AL** (FLcDNAs 4S-1, 4L-1 co-localized with *Acc-2* probe)	3 sequences (TA763, Lebanon)	(Friebe and Gill 1996)
T. monococcum AA	TA2025 (Turkey)	6	**1AL** (NOR, FLcDNAs 1S-1, 1L-2, co-localized with Acc2 probe), **3AL** (FLcDNAs 3S-1, 3S-2, co-localized with *Acc-2* probe), **4AL** (FLcDNAs 4S-1, 4L-1 co-localized with (GAA)	3 sequences	(Friebe et al. 1990)
Ae. speltoides SS	TA2368 (Turkey)	5	**1SL** (NOR), no pair - heterozygous plant, **3SL, 5SL**,(GAA, arm ratio)	2 sequences	(Friebe and Gill, 1996; Friebe et al. 2000)
	TA2780 (Israel)	4	**3SL, 5SL** (GAA, arm ratio)	–	
Ae. tauschii DD	TA1691 (Unknown)	4	**3DL** (pAs1), **5DL** (NOR, pAs1, DAPI heterochromatic block on 5DS near the centromere)	**3DL, 5DL** 2 sequences	(Friebe and Gill 1996)
T.turgidum subsp. *dicoccoides* AABB	TA73 (Lebanon)	10	**3AL, 4AL, 1BL, 3BL, 5BL**	3 sequences	(Dedkova et al. 2007)
	TA1385 (Iraq)	8	**3AL, 4AL, 3BL, 5BL**	–	
	TA1392 (Israel)	10	**3AL, 4AL, 1BL, 3BL, 5BL**	2 sequences	
	TA84 (Turkey)	8	**3AL, 4AL, 3BL, 5BL**	2 sequences	
T.turgidum subsp. *dicoccum* AABB	TA10492 (Ethiopia)	8	**3AL, 4AL, 1BL, 3BL**	–	(Gill et al., 1991; Friebe and Gill 1996)

T.turgidum subsp. *durum* AABB	Langdon Durum (USA)	6	**3AL, 4AL, 3BL**	–	(Gill et al., 1991; Friebe and Gill 1996)
	TA2932 Kamut Durum (unknown)	6	The same as Langdon Durum		
	TA9137 cv. Bozak (Azerbaijan)	6	The same as Langdon Durum		
T.timopheevii A^tA^tGG	TA103 (Yugoslavia)	10	**$1A^tL$** (FLcDNA 1L-1 co-localized with *Acc-2* probe)**, $4A^tL$,** – 2 *Acc-2* foci, (FLcDNAs 4S-1, 4L-1 co-localized with *Acc-2* probe) **1GL, 3GL, 5GL,** (FLcDNAs 1S-1, 1L-2, 3S-2; 5S-1, 5L-1, co-localized with GAA)	3 sequences	(Badaeva et al. 1994)

***** On some chromosomal spreads it is possible to distinguish two sites of *Acc-2* probe hybridization on the same chromatid of a chromosome which can be the evidence of multiple copies of the gene present on the same chromosome (Supplemental figure 6).

Table 2. Oligonucleotide FISH probes and *Acc-2* pooled probe

#	Probe	Sequence (5'-3') and flourochrome label	Amount applied to preparation, ng	Sequence used to develop probes
1	6-FAM-GAA	6-FAM-AAGAAGAAGAAGAAGAAGAAGAAGAA	0.5	
	Cy5-GAA	Cy5- AAGAAGAAGAAGAAGAAGAAGAAGAA	5	
2	pAs1-1	6-FAM-CAAAACGGACAATCTCTTTCAAAGTATCAGG	mixture, 100 ng each	D30736 (Rayburn and Gill 1986; Nagaki et al. 1995), X76300 (Vershinin et al. 1994)
3	pAs1-2	6-FAM-TCAGAGTTCATTTGAAATGCTTTTCA		
4	NOR	6-FAM-TGGCGCGCGTCAACTTCCGTC	100	X07841 (pTa71) (Barker et al. 1988)
5	pTa71	Fluorescein labeled plasmid with 9kb insert	100	(Gerlach and Bedbrook 1979)
6	5Sg	6-FAM-CTACTCTCGCCCAAGCACGCTTAAC	mixture, 200ng each	AY841019, AY841026, AJ409522, AY316207, FJ882469
7	5Ssp	6-FAM-TGTTACCCCCGTCTTCGTCCCTTAT		FJ882469, AJ409522
8	*Acc-2* (pooled)	RT PCR Primers:		
1c	1950bp	F ACAATGGGATGGCTGCGGTCAAG R CATCCGCGACCAAAAACCGAAGAA	mixture, 150ng each	U10187
2c	1942bp	F CAACCCGACTCCCGAAAGAT R GGGTCCTCCACTTCTCGGTAAATA		U10187
3c	2078bp	F GCAGAATCTAACGAGCATAACCAG R GTTTCAGCGAGATGCGACA		U10187

Table 3. Full length cDNA FISH probes used for chromosome identification

FISH probe	FLcDNA, KOMUGI database	Matching EST	EST position on bin map	Position of FISH signal on chromosomes of cv Chinese Spring	cDNA/probe length, bp
1L-1	tplb0013a02	BF482555	C-1BL6-0.32	1AL, 1BL, 1DL centromeric	5094
1L-2	tplb0029f23	BE591501	1A? 1BL6-0.32-0.47 1DL2-0.41-1.00	1AL, 1BL, 1DL distal end	3113
1S-1	tplb0048d21	BE425354	1AS1-0.47-0.86 1DS3-0.48	1AS, 1BS, 1DS centromeric	3487
3S-1	tplb0011e24	BF200563	3AS4-0.45-1.00 3BS1-0.33-0.57 3DS3-0.24-0.55	3AS, 3BS, 3DS interstitial	1491
3S-2	tplb0004j16	BF202364	3AS4-0.45-1.00 3DS3-0.24-0.55	3AS, 3BS, 3DS interstitial	4402
4S-1	tplb0017g02	BF485337	C-4AL11-0.66* 4DS2-0.82-1.00	4AL in the middle, 4BS, 4DS distal end	3191
4L-1	tplb0033b21	BE637255	4AS3-0.76-1.00 4BL1-0.71-0.86 4DL9-0.31-0.56	4AS distal end, 4BL, 4DL interstitial	3024
5L-1	tplb0043p15	BF483487 BE404437 BF474029	5DL5-0.76-1.00 5AL10-0.57-0.78	5AL, 5BL, 5DL distal end	1514
5S-1	tplb0027f03	BF482732	3AL3-0.42-0.78 3BL2-0.22-0.50 3DL2-0.27-0.81	5AS interstitial, 5BS, 5DS distal end	2416

Figures

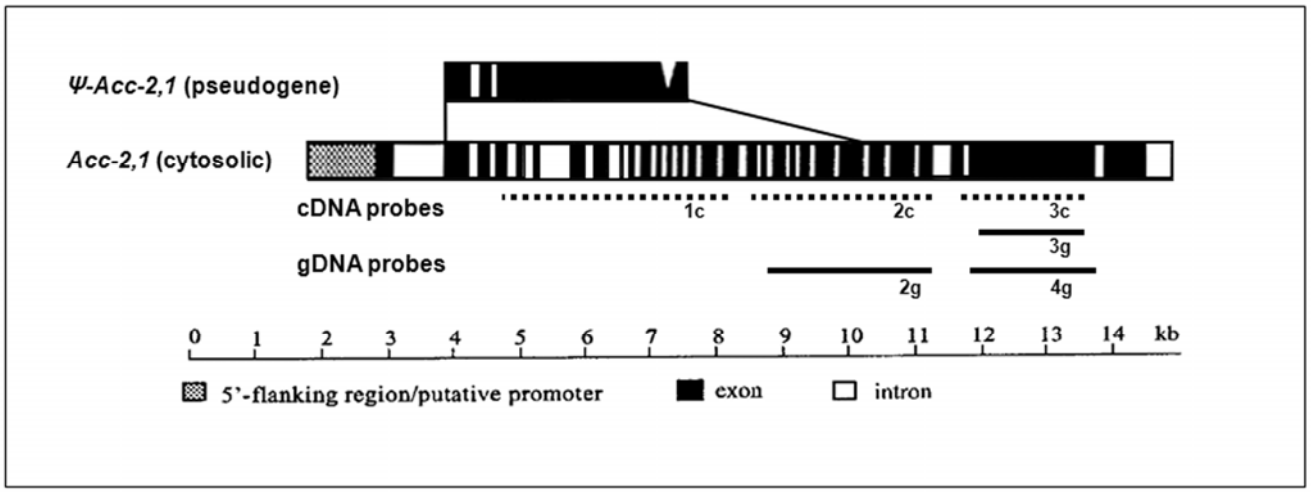


Figure 1 Positions of *Acc-2* FISH probes developed based on cDNA and gDNA sequences. The structure of the *Acc-2* gene and pseudogene is shown (adapted from (Faris et al. 2001)); cDNA probes were produced by RT PCR, gDNA probes were produced by PCR with gDNA as a template.

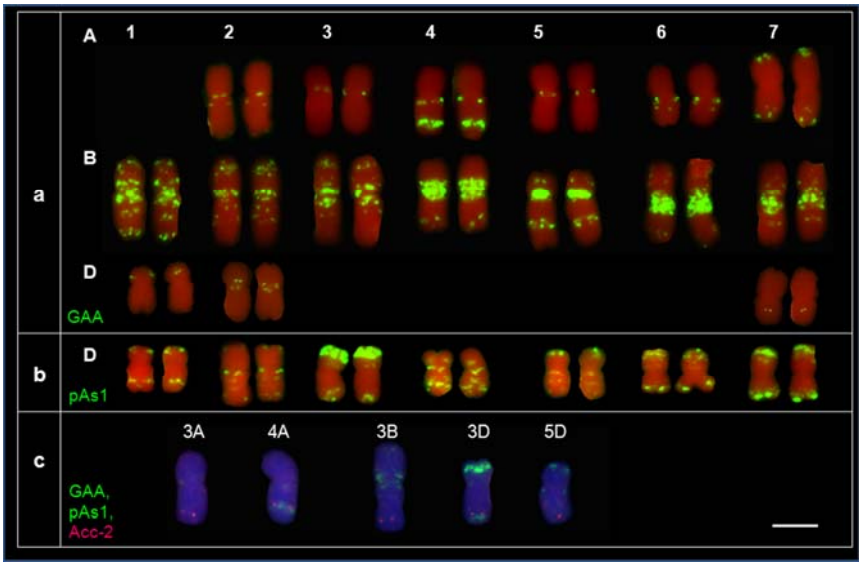


Figure 2 a, b FISH karyotype of cv Chinese Spring : **a.** GAA-oligonucleotyde probe pattern on A-, B- and D-genome chromosomes; **b.** pAs1-oligonucleotyde probe pattern on chromosomes of D genome; **c.** mapping of *Acc2* gene on wheat chromosomes. The *Acc-2* probe is red, GAA and pAs1 repeats are green, chromosomes are counterstained with DAPI (blue); bar corresponds to 5μm.

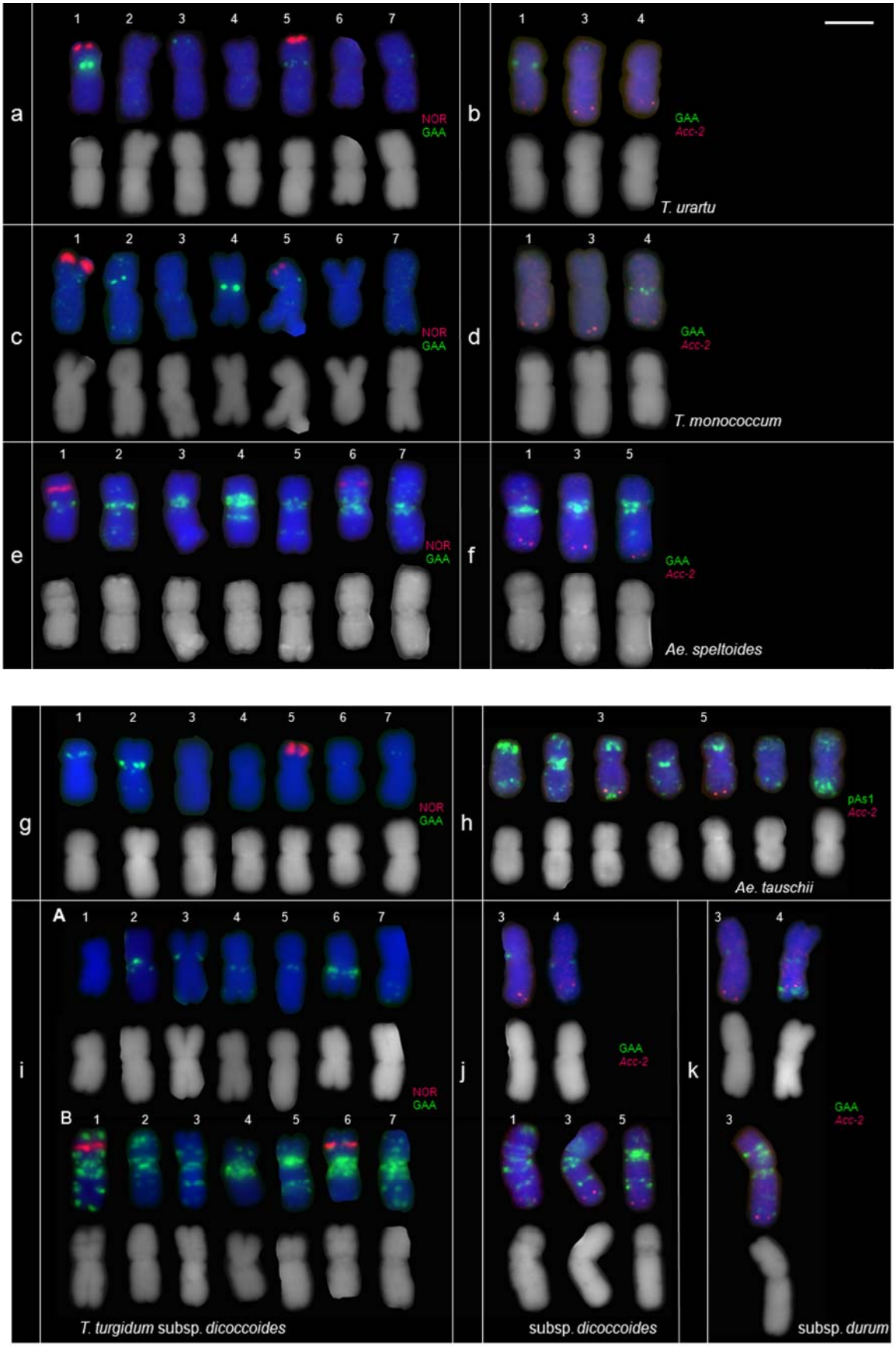


Fig. 3 (continued)

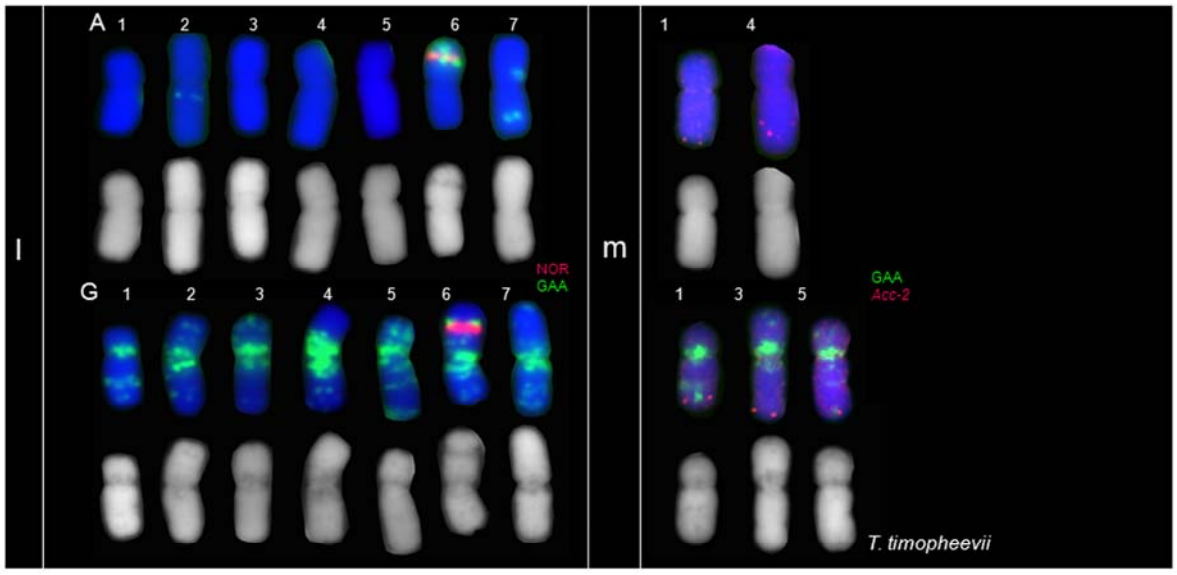


Fig. 3 (continued)

Figure 3 Identification of chromosomes of wheat species and mapping *Acc-2* sequences using FISH with GAA and NOR probes. Each panel presents merged images of chromosomes and DAPI channel only. Left panels present karyotypes with GAA probe in green and NOR probe (pTa71) in red. Right panels present chromosomes with *Acc-2* signals (red) and GAA (green), except panel **h**, where green is pAs1 repeat.

a, b - *T. urartu*; **c,d** – *T. monococcum* ; **e,f** – *Ae. speltoides* (TA2368), bar corresponds to 5μm; **g,h** – *Ae. tauschii*; **i,j** – *T. turgidum* subsp. *dicoccoides* (TA73), **k** – *T. turgidum* subsp. *durum*(Langdon Durum); **l,m** – *T. timopheevii*.

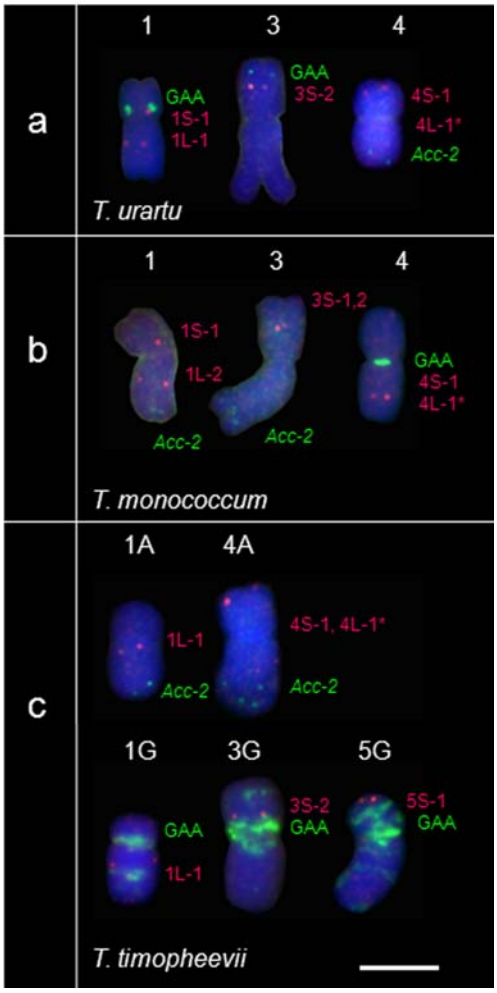


Figure 4 Identification of chromosomes of wheat species using FLcDNA probes.

a. *T. urartu*, probes: ch. 1: GAA - green, FLcDNAs probes 1S-1, 1L-2 - red; ch. 3: GAA - green, FLcDNA 3S-2 - red, ch. 4: FLcDNAs 4S-1, 4L-1 - red, *Acc-2* - green.

b. *T. monococcum*: ch. 1: FLcDNAs 1S-1, 1L-2 - red, *Acc-2* green, ch. 3: FLcDNAs 3S-1, 3S-2 - red, *Acc-2* green; ch. 4: GAA green, FLcDNAs 4S-1, 4L-1 - red.

c. *T. timopheevii* : ch. 1A: FLcDNA 1L-1 is red, *Acc-2* is green; ch. 4A: FLcDNAs 4S-1, 4L-1 are red, *Acc-2* is green; chromosomes 1G, 3G, 5G: GAA is green, FLcDNAs 1S-1, 1L-2; 3S-2; 5L-1, 5S-1 respectively, are red; bar corresponds to 5μm. * Probes 4S-1 or 4L-1 where applied together. Because it is not known whether chromosome 4A of these three species has inversions, we can not say which of the probes produce signal on the long or short arm.

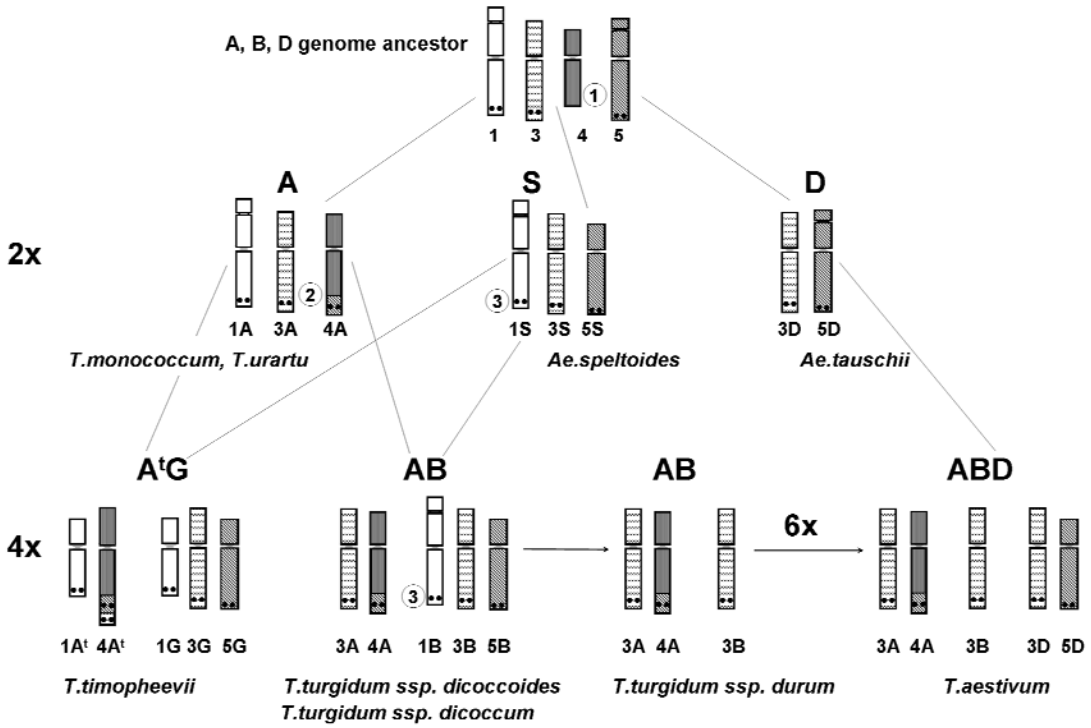
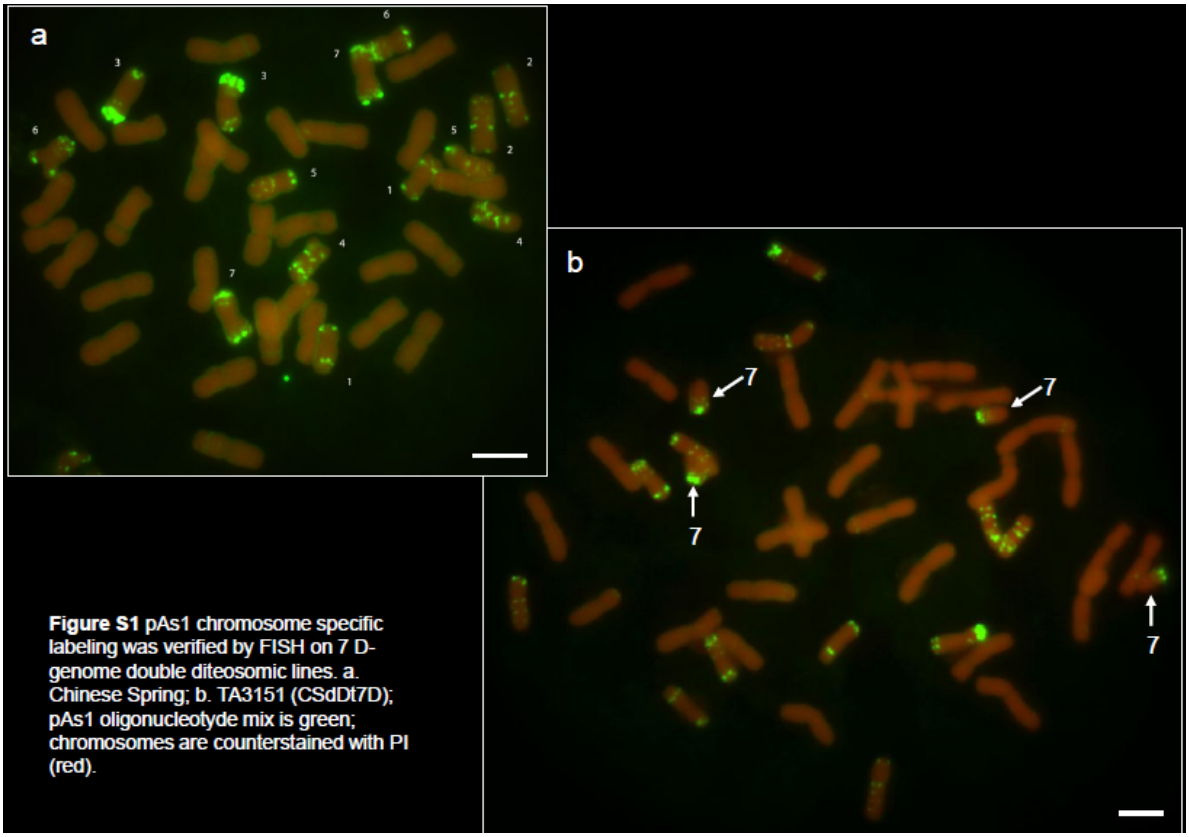
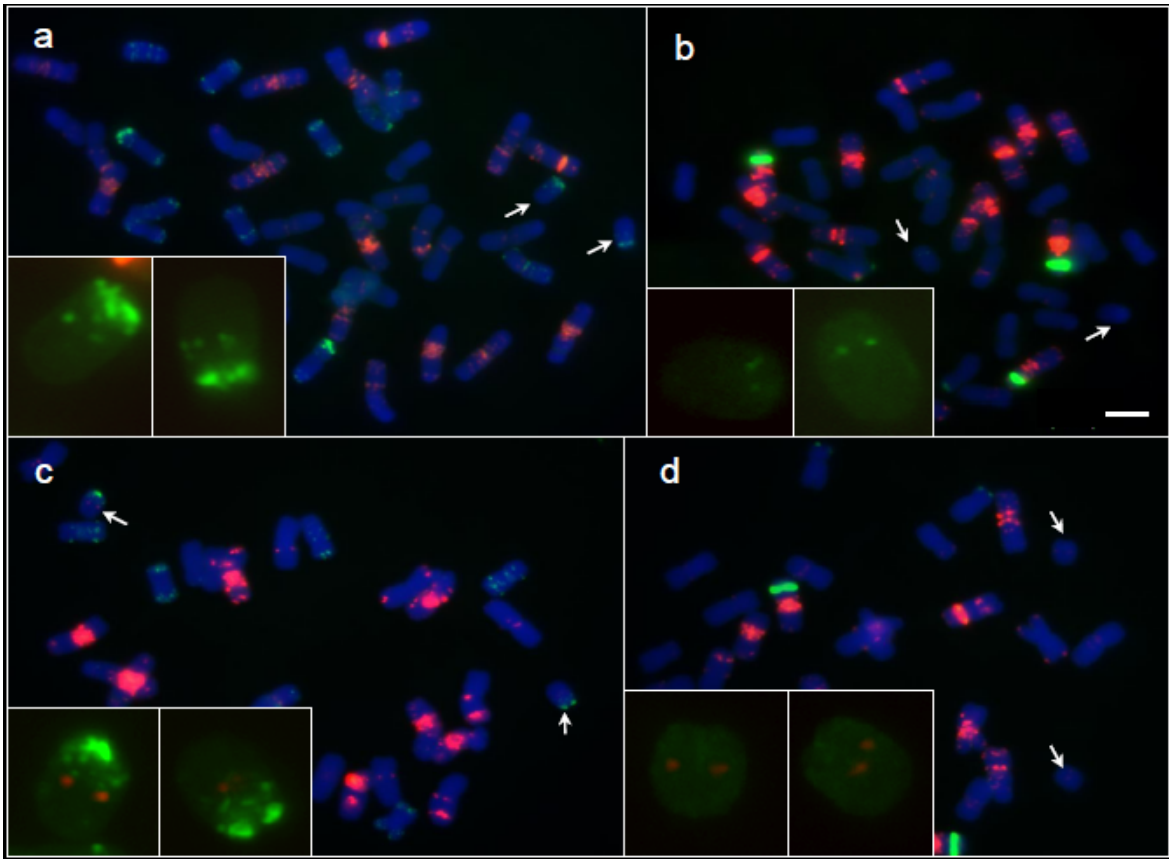


Figure 5 Chromosome modifications in wheat and *Aegilops* species as revealed by *Acc-2* FISH mapping. **1**. A translocation 4AL/5AL occurred at A-genome ancestor and present in A genomes of all species studied. **2.** 3AL/4AL translocation present in tetraploids of group Timopheevii. **3.** S-genomes and B-genomes are polymorphic in presence / absence of *Acc-2* foci on chromosome arm 1L and 5L. Solid dots represent locations of *Acc-2* foci. NORs are shown on chromosomes 1 and 5.

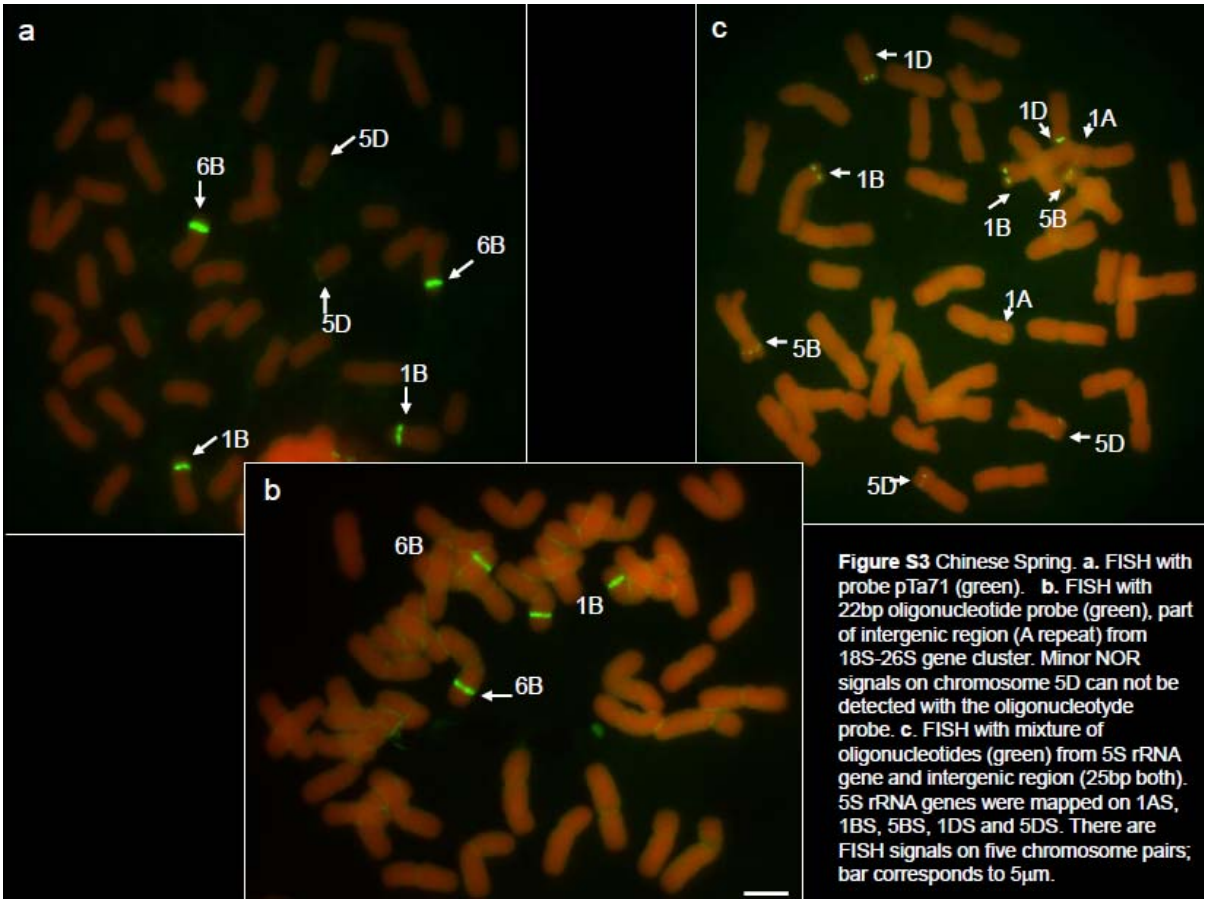


Supplemental figure 1 pAs1 chromosome specific labeling was verified by FISH on 7 D-genome double ditelosomic lines. **a.** Chinese Spring; **b.** TA3151 (CSdDt7D). Green – pAs1 oligo mix; chromosomes are counterstained with PI (red); bar corresponds to 5µm.



Supplemental figure 2 Identification of 7DS and 7DL chromosome arms using ditelosomic lines originated from Chinese Spring. Telosomes are shown with arrows. The physically shorter arm of chromosome 7D is homeologous to the long arms of chromosomes 7A and 7B (Werner et al., 1992). To verify GAA and pAs1 arm specific FISH pattern for chromosome 7D, two aneuploid ditelosomic lines originated from cultivar Canthatch and Chinese Spring were used. **a.** On both aneuploids genetically short arm of chromosome 7D has major distal pAs1 band and distinguishable minor pAs1 signal in the middle of the arm. **b.** As it was reported before (Friebe et al., 1996), 7DS arm has minor NOR signal which was confirmed by FISH with pTa71 probe. **c,d.** Genetically long arm of 7D also has major distal pAs1 band, no distinguishable minor pAs1 signals in the middle of the arm and no NOR signals, but there is a minor GAA signal in the middle of the arm.

a, b TA3130 CS Dt7DS; **c, d** TA3071 CS Dt7DL. Probes: GAA is red; **a, c.** pAs1 is green; **b, d** pTa71 is green; bar corresponds to 5µm.



Supplemental figure 3 Chinese Spring. **a.** FISH with probe pTa71 (green). **b.** FISH with 22bp oligonucleotide probe (green), part of intergenic region (A repeat) from 18S-26S gene cluster. Minor NOR signals on chromosome 5D can not be detected with the oligonucleotyde probe. **c**. FISH with mixture of oligonucleotides (green) from 5S rRNA gene and intergenic region (25bp both). 5S rRNA genes were mapped on 1AS, 1BS, 5BS, 1DS and 5DS. There are FISH signals on five chromosome pairs; bar corresponds to 5µm.

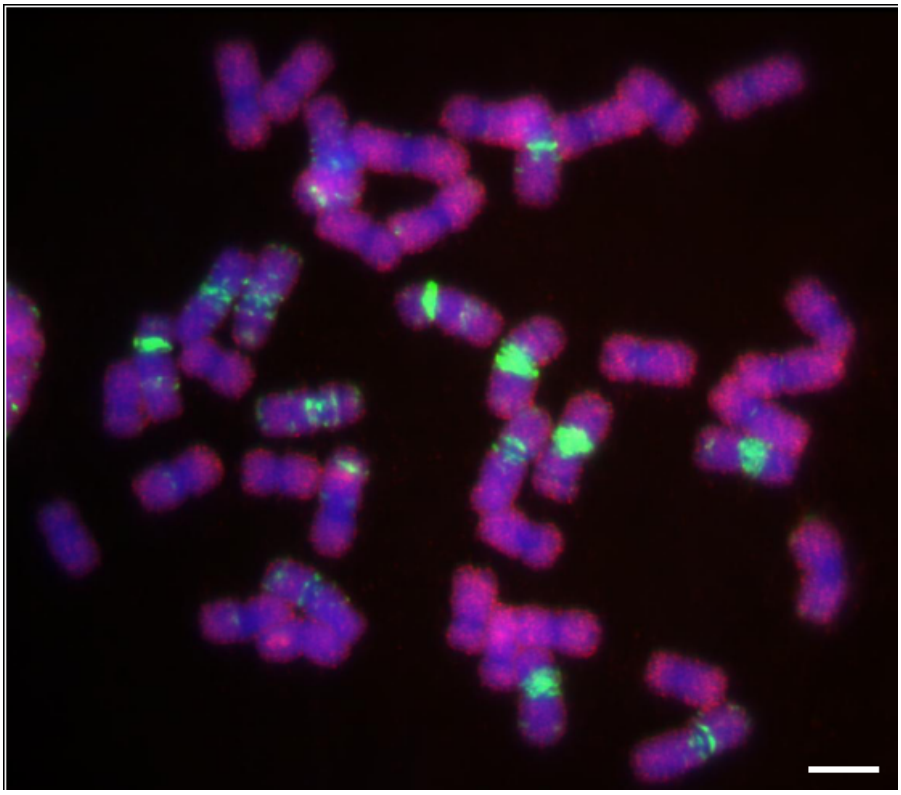


Figure S4 FISH with *Acc-2* gDNA probe 2g (red) produce staining on all chromosomes; GAA oligo probe is green; Chinese Spring; bar corresponds to 5μm.

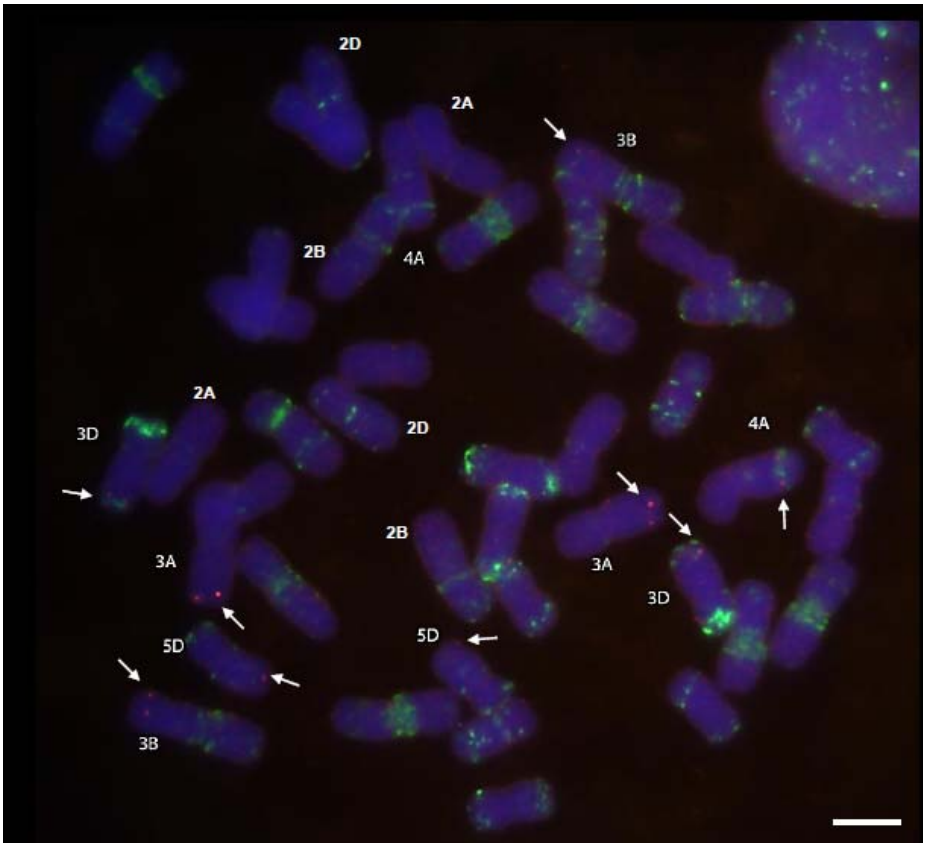


Figure S5 FISH with *Acc-2* cDNA pooled probe (6kb) on metaphase spread of cv. Chinese Spring. *Acc-2* probe is red, GAA and pAS1 repeats are green; chromosomes are counterstained with DAPI (blue). *Acc-2* signals are present on the long arms of chromosomes of group 3, 4AL and 5DL; bar corresponds to 5µm.

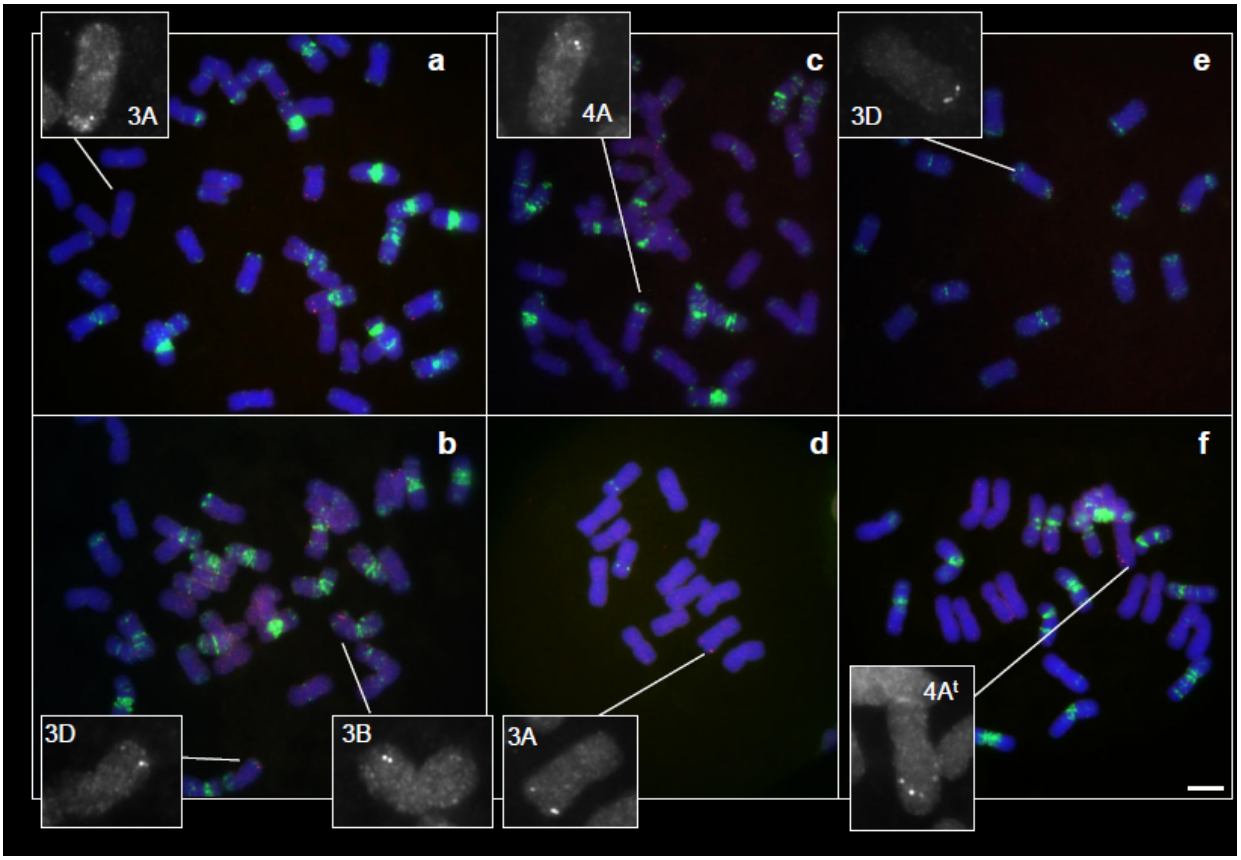


Figure S6 Double signals of *Acc-2* probe (red). *T. aestivum*: **a** Chinese Spring, **b** Wichita, **c** TAM107; **d** *T. urartu*, **e** *Ae. tauschii*, **f** *T. timopheevii.* Green oligonucleotide probes are: **a-c** GAA and pAs1, **e** pAs1, **d,f** GAA; bar corresponds to 5µm.

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