Molecular cytogenetics and genomics of novel wheat-*Thinopyrum bessarabicum* recombinant lines carrying intercalary translocations

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Abstract

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The diploid wild grass *Thinopyrum bessarabicum* (2*n* = 2*x* = 14, JJ or E^bE^b) is a rich source of important genes for bread wheat (2*n* = 6*x* = 42) improvement because of its salinity tolerance and disease resistance. Development of wheat–*Th. bessarabicum* translocation lines by backcrossing amphiploids in the absence of the *Ph1* gene (allowing intergenomic recombination) enables its practical utilization in wheat improvement. Using genomic *in situ* hybridization (GISH) and repetitive probes for fluorescent *in situ* hybridization (FISH), six novel wheat–*Th. bessarabicum* translocation lines involving different chromosome segments (T4BS.4BL-4JL, T6BS.6BL-6JL, T5AS.5AL-5JL, T5DL.5DS-5JS, T2BS.2BL-2JL, and the whole arm translocation T1AL.1JS) were identified and characterized in this study. No background translocations between wheat genomes were observed. The involvement of 5 of the 7 chromosomes, and small terminal segments of the *Th. bessarabicum* chromosome arm were important, contributing to both reduced linkage drag of the derived lines by minimizing agronomically deleterious genes from the alien species, and high stability including transmission of the alien segment. All three wheat genomes were involved in the translocations with the alien chromosome, and GISH showed the *Th. bessarabicum* genome was more closely related to the D genome in wheat. All the introgression lines were disomic, stable and with good morphological characters. The work also generated a high-resolution karyotype of two accessions of *Th. bessarabicum* using multiple repetitive DNA probes for chromosome identification. A complete CS-*Th. bessarabicum* amphiploid (2*n*=8x=56, AABBDDJJ) was used and each individual J-genome unambiguously identified. The established karyotype will be useful for the rapid identification of potential donor chromosomes in wheat improvement programs, allowing appropriate alien-chromosome transfer. Genotyping-by-sequencing (GBS) data was collected from the wheat-*Th. bessarabicum* introgression lines, but the complexity of the wheat genome and need for further development of data analysis pathways limited interpretation.

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I would like to start with British author James Allen's quote "*No duty is more urgent than that of returning thanks*."

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Abbreviations

AFLP	Amplified fragment length polymorphism
BAC-FISH	Bacterial artificial chromosome fluorescence *in situ* hybridization
BC_1	first backcrossed generation
BAC	Bacterial Artificial Chromosome
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	Base pairs
CS	Chinese Spring
CTAB	Cetyltrimethylammonium bromide
DAPI	4',6-diamidino-2-phenylindole
dUTP	Deoxyuridine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediammine tetra- acetic acid
EST	Expressed sequence tag
FISH	Fluorescent *in situ* hybridization
GISH	Genomic *in situ* hybridization
HCl	Hydrochloric acid
IRAP	Inter-retroelement insertion polymorphism
M	Molar
McGISH	Multicolour fluorescence *in situ* hybridization
MYA	Million years ago
NOR	Nucleolar organizer region
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PVP	Polyvinylpyrilidone
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Rotations per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
SSC	Saline sodium citrate
SSR	Simple sequence repeat
Wt	Weight
v/v	Volume added to volume
w/v	Weight added to volume

Dedication

To all good people who have been part of this journey

पार्थ नैवेह नामुत्र विनाशस्तस्य विद्यते।
न हि कल्याणकृत्कश्चिद् दुर्गतिं तात गच्छति।

"No one who does good work will ever come to a bad end, either here or in the world to come"

----The Bhagavad Gita
Chapter 6 verse 40
(120 Mya old book)

Publications

Referred journal articles

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Chapter 1: General Introduction

1.1 Wheat and global food security

Wheat, the 'King of cereals', is amongst the most valuable staple food crops in the world, ranked behind only maize (*Zea mays* L.) and rice (*Oryza sativa* L.) with annual production over 722.2 million tonnes occupying 17% (219 Mha) of the average cultivated area worldwide (FAOSTAT, 2014). Nearly half of the world population depends on wheat as their primary food crop: wheat is part of the daily diet of more than 75% of the world's population. With around 148 million tones export quantity; it holds a prominent position in the international food grain trade (FAOSTAT, 2014). The global wheat trade per annum is higher than that of maize and rice combined. Wheat provides an ample source of energy for the world population, providing 20% (one fifth) of total food calories and protein in human nutrition, making it a commodity that remains to this date a central pillar of food security (FAO, 2011).

A number of factors will jeopardize global food security over the next 40 years. The global population has increased from 3 billion to 7 billion in last 50 years and is estimated to increase by 1 billion over the next 12 years to reach 9.6 billion by 2050. Virtually, population growth will be mainly in developing countries, with more than 55% in Asia by 2050 (FAO, 2009). With an increasing global population and changes in diet, the demand for food is predicted to increase about 40% by 2030 (Dixon, 2009). To meet this demand from a much larger, wealthier and more urban population, food production worldwide will have to increase by 70% (FAO, 2009). Demand for wheat is predicted to increase in the future as the global population increases. Wheat production will have a critical role in global food security and the agriculture economy in the coming years. To meet expected demand, wheat breeders and farmers around the world will have to increase wheat production using limited natural resources, like fertilizers, pesticides, water and land. The world wheat scientific community is endlessly working to increase wheat production by exploiting wild genetic diversity and utilizing important traits and genomic resources. However, wheat production is challenged by the global climate change, water scarcity and salinity, soil loss, emerging pathogen species and rising energy costs for agronomy.

Novel germplasm will be useful in breeding programs to create new crops and new uses for existing crops, to meet breeding objectives for sustainability of crop production, and to ensure the entire world's people benefit from crop improvement through enhanced food security and quality (Heslop-Harrison, 2002).

1.2 Origin, domestication and evolution of wheat

Domestication is the outcome of the genetic selection process that, by altering key traits, transforms wild forms into cultivated varieties of crops and animals (Nesbitt, 2001, Salamini et al., 2002). Domesticated species are dependent on human intervention for their reproduction, nutrition, health, planting, and dispersal (Heslop-Harrison and Schwarzacher, 2012). It is still being questioned whether selection pressure applied consciously by humans or it was the result of human cultivation and rearing of animals (Gepts, 2004, Harlan et al., 1973, Zohary et al., 1998).

Domestication is associated with genetic bottlenecks, resulting in reduced genetic variability. Genetic analysis has shown that many of the gene alleles involved in the domestication syndrome are present within the gene pool of wild progenitors of crops although with a low frequency, while other traits are apparently new mutations breeding of new plant varieties require genetic variation. This can come from wild collections of germplasm (Heslop-Harrison and Schwarzacher, 2012, Heslop-Harrison, 2002). Domesticated plant species are found in 160 taxonomic families: out of 400,000 species of flowering plants about 2,500 species are estimated to have undergone domestication, 250 species are considered as fully domesticated as food and feed plants and just 12 species provide 75% of the food eaten (Dirzo and Raven, 2003, Gepts, 2012, Heslop-Harrison and Schwarzacher, 2012, Meyer and Purugganan, 2013).

Wheat was one of the first domesticated food crops. The first domestication of wheat happened approximately 10,000 years ago in the Fertile Crescent, as a part of ‘Neolithic Revolution’, which was a landmark in humankind’s transition from a lifestyle of hunting and gathering of food to settled agriculture (Diamond, 2002, Doebley et al., 2006, Shewry, 2009, Zohary et al., 2012). The diploid wheat Einkorn (*T. monococum*) was the first wheat species to be widely grown and domesticated ~10000 years ago in the Karacadag mountain range in southeastern Turkey (Heun et al., 1997, Peng et al., 2011).

This site was identified from the analysis of 288 AFLP marker loci in einkorn and its wild ancestor (Feuillet et al., 2008, Harlan and Zohary, 1966, Heun et al., 1997, Vasil, 2007). The domesticated Einkorn varies from wild *T. boeoticum* in seed and spikelet size. The wild Einkorn wheat has bigger seed and spikelet as compared to domesticated Einkorn wheat.

A further important step in the evolution of modern polyploid wheat varieties was the domestication of emmer, which is tetraploid wheat, from its wild progenitor *T. dicoccoides* (Körn. ex Asch. & Graebner) Schweinf. Wild emmer, AABB wheat with its A-genome from *T. urartu* Tumanjan ex Gandilyan, has brittle ears that shatter at maturity into spikelets, which results in seed loss at harvesting. Loss of seed shattering was a key event in the domestication of major cereals (Doust, 2007, Harlan, 1992, Hammer, 1984, Konishi et al., 2006). Unlike their wild progenitor, all domesticated tetraploid wheat has a non-brittle rachis; the transformation of the brittle rachis to non-brittle could be the first symbol of domestication in wheat (Peng et al., 2003). Domesticated emmer wheat, *T. dicoccum*, has hulled seeds (presence of kernels in the free-threshing (naked) form.

The latest step in the *Triticum* domestication was free-threshing wheat - tetraploid pasta wheat (*T. durum*) and hexaploid bread wheat (*T. spelta* and *T. vulgare*). There have been a lot of discussions whether the first hexaploid wheat was a hulled or free-threshing. Recently Dvorak et al. (2012) proposed that the ancestral hexaploid wheat was hulled and resulted from a cross between free-threshing tetraploid wheat, not hulled cultivated emmer wheat, and the strangulata subspecies of *Ae. tauschii*. To understand the origin of free-threshing wheat, it is necessary to have knowledge of how hexaploid wheat is evolved. Both *T. spelta* and *T. vulgare* has an AABBDD hexaploid genome, although neither species is found in the wild except as a transient escape (Salamini et al., 2002, Feldman and Kislev, 2007, Feldman and Millet, 2001).

The amount of the literature available on the history and evolution of the wheat suggests its potential role in agricultural economies and human civilization (Nesbitt, 2001). Wheats are cereal grasses of the *Graminae* (*Poaceae*) family and of the genus *Triticum*, which contains over 500 species in at least 23 genera (Baum et al., 2013). Wheat is a very recent crop in evolutionary terms, today, about 95% widely grown modern wheat cultivars are hexaploid bread wheat (*Triticum aestivum)* and another 5% being

tetraploids pasta wheat (*T. turgidum)* (Shewry 2009). Present day commercial wheat is a product of long and interesting evolutionary process.

Diverse polyploid wheat species were established inside the *Triticum* genus near about ~7 million years ago. There were two diploidization events that led to the development of bread wheat. The first diploidization event created the tetraploid wheat when wild diploid wheat *T. urartu* (AA genome) hybridized with a yet unknown species from the B genome ancestor closely related to goat grass *Aegilops speltoidies* (SS genome) 300,00-500,00 years ago, which was domesticated ~10000 years ago and became known as emmer wheat (*T. turgidum*) (Fig. 1.1). A second diploidization event that led to bread wheat lineage happened between tetraploid emmer wheat and the wild diploid species *Aegilops tauschii* (DD genome) giving rise to fertile hexaploid wheat (AABBDD genome) now known as *T. aestivum* or bread wheat (Fig. 1.1).

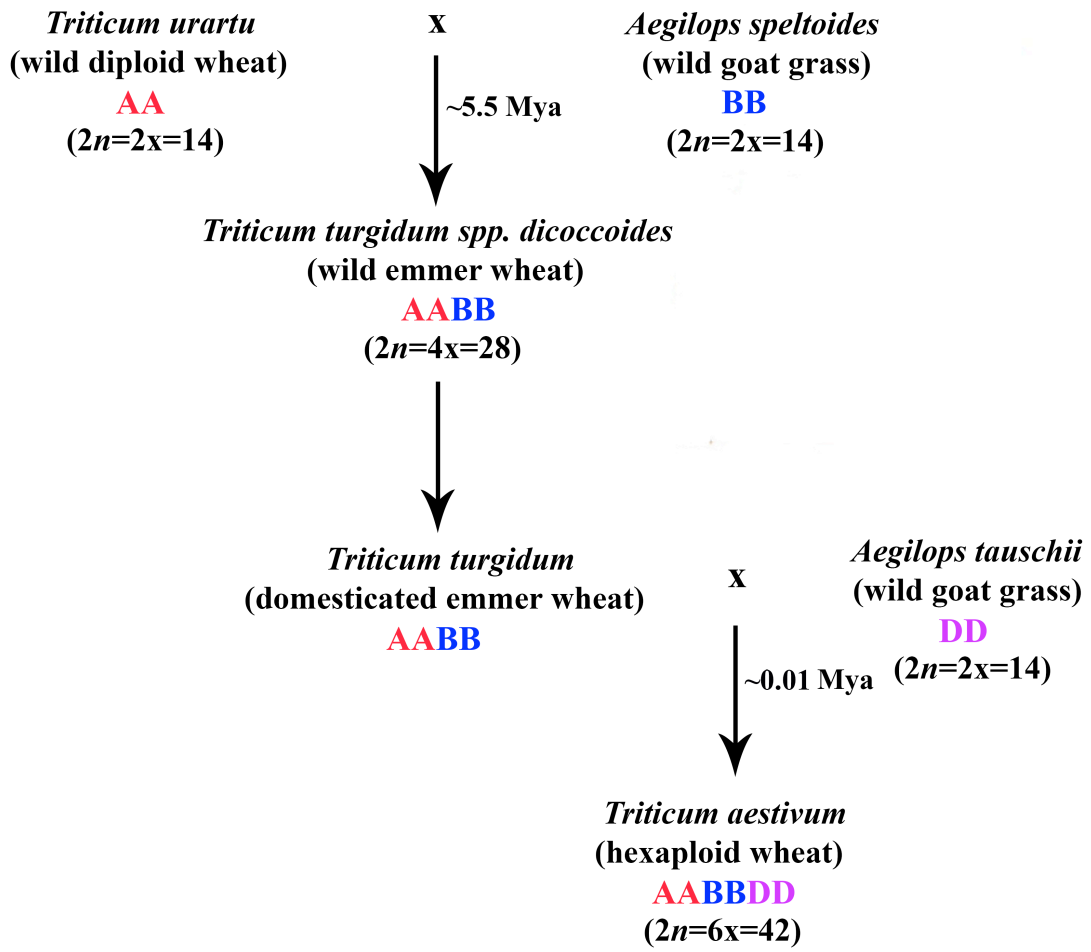


Fig. 1.1: Evolution of the wheat, modified from Feldman et al. (1986), Sears (1950), Kihara (1944).

1.3 Cytogenetics and genomics of wheat

1.3.1 The wheat genome

Bread wheat (*Triticum aestivum* L.) is a disomic allohexaploid (2*n*=6*x*=42) and has three subgenomes A, B and D (each subgenome has 7 chromosomes, making *n*=21) that are assigned to seven homoeologous groups (Fig. 1.2), each homoeologous group has three closely related chromosomes, one from each of the three related subgenomes (Riley, 1958; Sears and Okamoto, 1958). The chromosome size and gene content varies within homoeologous sets of chromosomes; 3B is the largest wheat chromosome while 1D is the smallest one (Furuta et al., 1986).

The wheat genome is the Mount Everest of plant genomics. The genome of wheat is large and complex (16,000 Mb) with >80% repetitive sequences, which is 5, 535 and 110 times higher than human (3000 Mb), maize (2500 Mb) and rice (389 Mb) respectively (Smith and Flavell, 1975, Faris et al., 2002). The wheat D-genome is approximately 10-fold larger than that of rice and twice that of the maize genome (Arumuganathan and Earle, 1991). It is estimated that hexaploid wheat has 94,000 to 96,000 genes, which is almost close to the previous estimates based on the chromosome 3B, overall up to 50,000 genes per diploid genome (Brenchley et al., 2012, Choulet et al., 2010). In a recent study, it was found that a major part of the wheat genome is composed of transposable elements (TEs), which include highly repeated families and sequences (Choulet et al. 2010). The largest wheat chromosome is 3B (~1 Gb). It was the first chromosome for which a BAC library was constructed and a physical map achieved (Choulet et al., 2014).

After the release of the Arabidopsis genome in 2000 (Arabidopsis Genome Initiative 2000) and the advent of Next Generation Sequencing (NGS) technology in 2005, the number of sequenced plant genomes has rapidly increased to more than 100 (Michael and Jackson, 2013). Several genomes have been sequenced in the *Poaceae* family: Rice (389 Mb) (The International Rice Genome Sequencing Project 2005), Sorghum (730 Mb) (Paterson et al., 2009); Maize 2500 Mb (Schnable et al., 2009); Brachypodium 355 Mb (Vogel et al., 2010); Panicum 1.4 Gb (Casler et al., 2011). Other members of the tribe *Triticeae* to have been partially sequenced include barley, (without its complete repetitive intergenic regions, 5.1 Gb; The International Barley Genome Sequencing Consortium 2012) and rye (estimated to be 8.1 Gb) (Doležel et al., 1998).

Even with today's advancement of the next generation sequencing technologies, the limited information about the complexity and organization of the hexaploid wheat genome sequence obstructs the delimitation of the most cost-effective and informative sequencing technology (Metzker, 2010). Sequencing the bread wheat genome has always posed a challenge due to the size and complexity, its allohexaploid (2n = 6x = 42, AABBDD) nature and high content of repetitive DNA (Bennetzen et al., 2005, Devos et al., 2005, Smith and Flavell, 1975). To sequence the wheat genome, the International Wheat Genome Sequencing Consortium (IWGSC) was established in 2005 by a group of wheat growers, plant scientists, public and private breeders (http://www.wheatgenome.org). The IWGSC has more than 1000 members in 57 countries as a part of an international collaborative consortium. To reduce a complexity of the analysis, IWGSC has been using chromosome-based approach (Doležel et al., 2009). Key to this "chromosome-by-chromosome" strategy was the ability to individually flow sort nearly all the chromosomes of hexaploid wheat variety 'Chinese Spring' for which extensive genetic stocks, nulli-tetrasomics, and deletion lines were available. More recently, Berkman et al. (2011) carried out direct sequencing of a flow-sorted wheat chromosome arm 7DS, though the assembly is fragmented and relied greatly on the conservation of synteny for ordering of contigs. The first milestone was achieved in July 2014 when the first chromosome based draft sequence was published in the journal *Science* by IWGSC. Sequencing for the wheat genome is still underway (http://www.wheatgenome.org).

1.3.2 Wheat chromosome structure

Mendel's laws of inheritance led to the discovery of genes, which are located within the chromosomes. This paved pathway to the birth of classical cytogenetics (Peters, 1959, Sutton, 1903, Darlington, 1929). The studies soon extended in plants and animals leading to diverse genetic information. The wheat cytogenetics research began in 1918 with an understanding that common wheat ($2n$=$6x$=42) has 42 chromosomes (Sakamura, 1918). Based on meiotic pairing, Kihara (1919) and Sax (1922) showed that different wheat species may be diploid, tetraploid, or hexaploid and they shared one genome (later designated A) in common. Later work showed that the second genome (later designated B) of tetraploid wheat was derived from a species similar to *Aegilops speltoides* Tausch. They also showed that tetraploid and hexaploid wheat had two genomes (A and B) in common. Kihara (1944) and Mcfadden and Sears (1946)

recognized that the D genome of bread wheat was donated by the diploid goatgrass *Ae. tauschii* Coss.

Wheat cytogenetics took a landmark step after the pioneering work of Ernie Sears on wheat aneuploidy, begun in the 1930s, which have seen a major transformation in the way scientists extract genetic information from a wheat genome. Sears (1954, 1960) developed mono-, tri- and tetrasomic cytogenetic stocks for all chromosomes and nullisomics for 11 chromosomes and 42 compensating nullisomic-tetrasomics. Monosomic lines were used to produce substitution that helped locating and linkage mapping of QTL on individual chromosomes while nulli-tetrasomics were used to measure chromosome homology based on sporophytic and gemetophytic compensation. The aneuploid stocks also were used to determine the genomic affinity of individual chromosomes. O'mara (1940) produced wheat-rye addition and substitution lines for comparative mapping. After the discovery of C-banding technique, which enabled identification of individual wheat and rye chromosome. The complete ideogram of 21 wheat chromosomes was established using C-banding (Endo, 1986, Gill and Kimber, 1974a, 1974b). Developments of advanced cytogenetic techniques (FISH, GISH and immunostaining) were groundbreaking discoveries and ignited a revolution in wheat cytogenetics (Gall and Pardue, 1969, John et al., 1969, Molnár-Láng et al., 2000, Schwarzacher et al., 1989). Fluorescent *in situ* hybridization using repetitive DNA sequences which gives unique banding pattern along the wheat chromosome (Fig. 1.2) has been useful in identifying and establishing FISH karyotype of all wheat 21 chromosome (see Fig. 1.2) (Badaeva et al., 1996, Badaeva et al., 2002, Kubaláková et al., 2005, Mukai et al., 1993a, Pedersen and Langridge, 1997).

1.3.3 Chromosome pairing in wheat

Despite the fact that wheat is hexaploid, it behaves like a diploid during meiosis (it forms 21 bivalents). This diploid like behavior is controlled by *Ph1* gene mapped and found to be located on the long arm of chromosome 5B (Sears 1976), which restricts homoeologous pairing so that only homologous partners can pair. *Ph1* activity is unique to 5B. It has been demonstrated that *Ph1* is not the only gene controlling meiotic chromosome pairing in wheat. Several other (*Ph2*) pairing suppressor genes on short arms of chromosomes 3A; 3B and 3D (Mello-Sampayo, 1971, Miller et al., 1983, Driscoll, 1973, Upadhya and Swaminathan, 1967) have also been reported.

A number of hypotheses have been put forward to explain the mode of action of *Ph1*. Riley (1960) proposed that *Ph1* shortens the period available for synapsis, so that homologues, but not homoeologue, have sufficient time to pair. According Upadhya and Swaminathan (1967) the absence of the *Ph1* locus reduces the degree and speed of chromosome condensation, which, in turn, would allow homoeologous pairing. The recent molecular analysis revealed that the *Ph1* activity is related to *Cdk-2* like genes in mammals and the *Ime2* gene in yeast. These kinases, as well as the related kinase *Cdc28*, have been shown to control premeiotic replication and the formation of double strand break, and to reduce the level of the synapsis protein *Hop1*, which is essential for correct associations during synapsis (Griffiths et al., 2006, Moore and Shaw, 2009). Such homology among the genes controlling premeiotic and meiotic procedures suggests conserved basic mechanisms of homologue recognition within plants, yeasts, and mammals.

An important genetic stock has been developed with the use of radiation treatment. Sears (1977) produced two mutant lines (*ph1b, ph1c)* in CS wheat with deletion at the *Ph1* locus of about 70 Mb (Dunford et al., 1995). The original *Ph1* locus was defined by a single deletion of 70 Mb containing over 200 genes, but after more recent studies, it has been delimited to a region of a 3Mb containing less than 7 genes (Roberts et al., 1999, Gill and Gill, 1996, Gill et al., 1993, Sears, 1977). In the homozygous *ph1b* genotypes, homoeologous pairing between wheat and alien chromosomes is possible. This stock has been widely used in chromosome engineering as a popular way to transfer alien genes to wheat, as discussed in section 1.5.

1.4 Repetitive DNA sequences in *Triticeae*

A very large fraction of the nuclear genome of higher plants consists of repetitive DNA sequences consisting of motifs between two and ten-thousand or more base pairs which are repeated hundreds or thousands of times, and are scattered throughout the genome (Britten and Kohne, 1968, Kamm et al., 1995, Kubis et al., 1998). All genomes contain repetitive DNA sequence as a major portion of their genome. However, the degree to which it occurs within genomes is highly variable (Sharma and Raina, 2005).

The tribe *Triticeae,* contains a group of 400-500 diploid and polyploid species (Melderis et al., 1980), and is characterized by large genome sizes ranging from 5500-17000 Mb. Repetitive DNA accounts more than 70-80% of their genome while the

single-copy DNA may account for less than 1% of the genomes (Leitch and Bennett, 2004, Smith and Flavell, 1975).

Repetitive DNA fraction has played a major role during polyploidization and post-polyploidization changes. They have been part of evolutionary genome transformation. A part of repetitive DNA (non-coding) plays a regulatory role, whereas the other part simply provides structural stability to the chromosomes. Studies have reported that changes in repetitive DNA cause chromosomal rearrangements; triggering the repetitive DNA change itself, hence through mechanisms of concerted evolution (Elder Jr and Turner, 1995, Feldman and Levy, 2005, Ma and Gustafson, 2005). Its is of significant importance to have the knowledge about the distribution of repetitive DNA sequences for the investigation of sequence relationships and is essential to gain insight into the organization, evolution and behavior of plant genomes (Harrison and Heslop-Harrison, 1995). It has been demonstrated that dispersed repetitive DNA elements are convenient landmarks for valuable aspects of genome analysis, such as chromosome walking (Nelson et al., 1989) and transcript isolation (Valdes et al., 1994). As is well recognized in many plants, heterochromatin is poor in genes and mainly consists of tandemly repeated DNA families. The numerous genome or species-specific repetitive DNA sequences have been isolated and characterized in the tribe *Triticeae*, including wheat.

1.4.1 pSc119

Bedbrook et al. (1980) first revealed the presence of tandemly repeated DNA sequences in rye, first among the cereals. The 120-bp repeated DNA sequence *pSc119* that was originally isolated in rye (Bedbrook et al., 1980), belongs to the Ty3-gypsy retrotransposon family, and is located in the major heterochromatin blocks of B-genome (Lapitan et al., 1986, Zhang et al., 2004a, 2004b). This sequence was cloned from rye as *pSc119* and was later sub-cloned as *pSc119.1*, *pSc119.2* and *pSc119.3* (Mcintyre et al., 1990) but sequence analysis confirmed only *pSc119.2* contains the 120 bp repeat unit sequence. Southern hybridization or dot blotting has shown that the sequence is widespread in the *Triticeae* (Ørgaard and Heslop-Harrison, 1994, Mcintyre et al., 1988). Later on *in situ* hybridization using this repeat unit gave a characteristic banding pattern, which allowed identification of all B-genome and some A-genome wheat chromosomes.

1.4.2 dpTa1/pAs1

Another repetitive DNA sequence pAs1, belonging to the Afa-family (Rayburn and Gill 1986; Nagaki et al. 1998) (Nagaki et al., 1998, Rayburn and Gill, 1986) were localized to the D-genome heterochromatin (Zhang et al. 2004b). Other DNA elements belonging to the Afa and CACTA transposon families were located in the subtelomeric regions of all 21 chromosomes of wheat and other *Triticeae* species (Zhang et al., 2004a, Li et al., 2004). A similar sequence, *pHcKB6* was found in *Hordeum chilense* (Anamthawat-Jonsson and Heslop-Harrison, 1993).

1.4.3 45S and 5S rDNA family

Ribosomal RNAs (rRNAs) are the key structural components of ribosomes. Their high repetitive nature makes them good chromosome markers, widely used in cytogenetics (Hasterok et al. 2001; Leitch and Heslop-Harrison 1992) (Hasterok et al., 2001, Leitch and Heslop-Harrison, 1992). The genes for 45S rRNA and 5S rRNA are clustered at small number of sites, organized in tandem arrays at one or more loci at the nucleolar organizer regions of chromosomes in the genome (Schmidt and Heslop-Harrison, 1998), in the Triticeae, occur in many thousands of copies, most of which are not expressed (Flavell et al., 1993). Because of the sequence variation between and within loci and the potentially large number of loci, the genes are difficult to map by RFLP or genetic methods. However, knowledge of the relative physical locations, the number of loci, and copy number at each locus are important and useful. In the Triticeae several repetitive probes corresponding to multigene, such as 45S (*pTa71*) and 5S rDNA (*pTa794*) have been cloned and used for identification specific chromosome involved in the alien introgression (Mukai et al., 1991, Schwarzacher et al., 1989). Wheat has four 45S rDNA major sites on the 1B, 6B, and minor site on the 5D chromosome; chromosome 5A and 1A has 5S rDNA sites (Mukai et al., 1990). Members of the Triticeae show wide variation in the number of 18S-25S rDNA sites; barley has six sites on six of its seven chromosome pairs (Leitch and Heslop-Harrison, 1992, Pedersen and Linde-Laursen, 1994), while *Psathyrostachys stoloniformis* has seven sites on five of its seven chromosome pairs (Ørgaard and Heslop-Harrison 1994). Similarly, rye has only one pair of 45S rDNA sites on the 1R chromosome (Leitch et al., 1992), *Ae. umbellulata* has only two sites, one at a satellite region of a chromosome and the other more terminal (Castilho and Heslop-Harrison, 1995).

1.4.4 GAA sequences

The GAA-satellite sequence was first isolated by Dennis et al. (1980) in the form of clone *pHVG38* from barley, which is the major component of the highly heterochromatic B-genome chromosomes. The hybridization pattern of the GAA-satellite sequence on barley chromosomes has previously been described (Pedersen and Linde-Laursen 1994). It is also present in high-copy number in *Hordeum*, *Dasypyrum*, *Aegilops*, *Elymus* species containing the H genome, and *Triticum* species containing a B-genome (Pedersen et al., 1996).

1.4.5 Other repetitive DNA elements

The plants with large genome usually have more repetitive DNA elements (Kidwell, 2002). In wheat, the vast majority of the repeats are widely dispersed all over the chromosomes (Zhang et al. 2004). Kishii et al. (2001) found centromere specific tandem repetitive sequences (Tail family) in common wheat, which was originally, located in *Leymus racemosus* (*Triticeae*). In a recent study, Garbus et al. (2015) characterized six novel LTR retrotransposon families, including three Copias, one Gypsy, and two TRIM LTR retrotransposons on wheat homeologous group 4 chromosomes.

More recently, Komuro et al. (2013) isolated several novel repeat families from the bread wheat genome including novel repeats from A-genome. The most helpful banding patterns were generated by *pTa535*, *pTa-713*, and *pT-86 (*homolog of pSc119 sequence). Among them, the probe *pTa535* produced the highest number of signals on the A-genome chromosomes, and banding patterns were chromosome-specific. This clone is a 342-bp tandemly repeated DNA sequence, showing ~ 80% homology with clone *pTa173*, a member of the *Afa/dpTa1/pAs1*- family (Komuro et al., 2013). Recently Badaeva et al. (2015) successfully used *pTa535* pattern to distinguish *T. monococcum*, *T. boeoticum* and *T. urartu*. Even though the large number repetitive DNA families have been identified some of them remain to be found and uncharacterized. As new DNA sequencing technology is taking over, a reference genome would be crucial for the identification and characterization of these families. Current projects in Leicester are using novel bioinformatics approaches to identify simple and complex repetitive DNA sequences within wheat genomic sequence data.

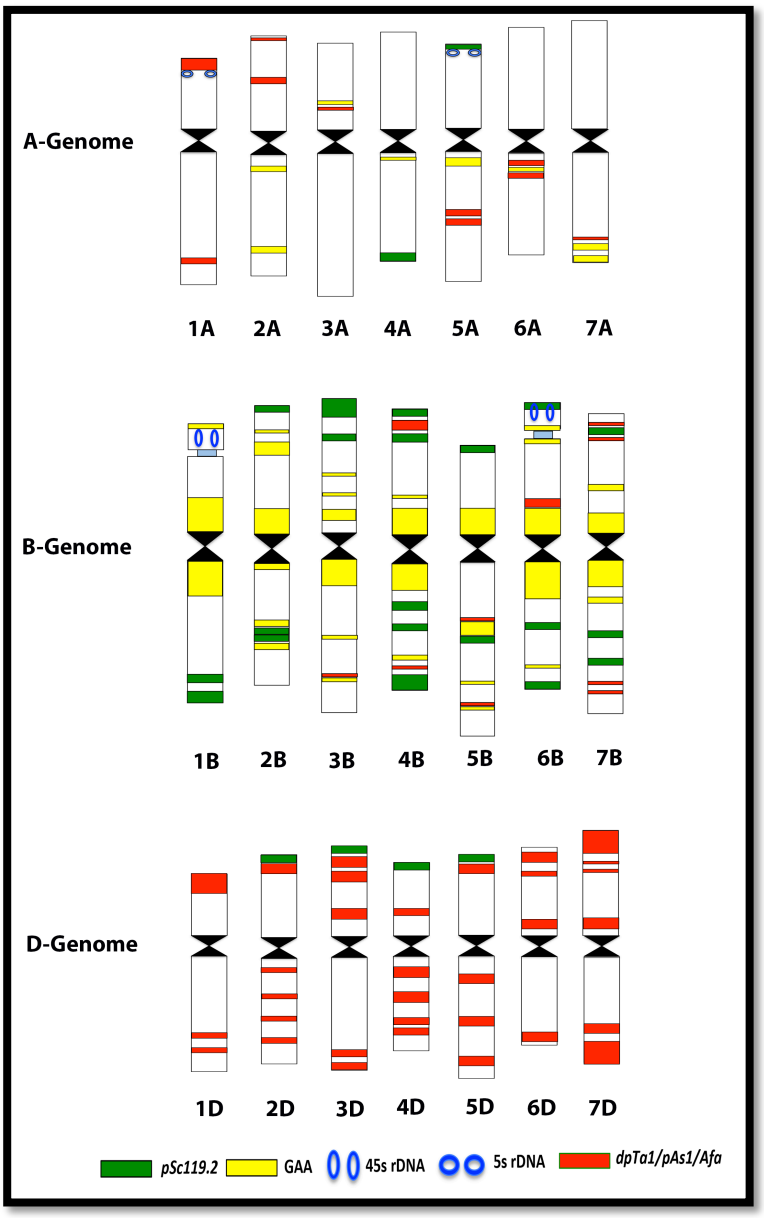


Fig. 1.2: Ideogram of Chinese Spring wheat showing genomic distribution of five repetitive DNA sequences: GAA (yellow), *pSc119.2* (green), 45SrDNA (blue), 5S rDNA (blue) and *dpTa1/pAs1* (red). Adapted from (Bardsley et al., 1999, Castilho and Heslop-Harrison, 1995, Danilova et al., 2012, Mukai et al., 1993b, Kubaláková et al., 2005, Sepsi et al., 2008, Cuadrado et al., 2000, Cuadrado et al., 2008).

1.5 Alien gene pool

According to gene pool concept of Harlan and De Wet (1971), which illustrates Vavilov's earlier recognition, there is a potential pool of genetic diversity available within each crop. The utilization and a measurement of that diversity depends on the relative crossing ability between the crop itself and the primarily non-domesticated species in the primary, secondary or tertiary gene pool of the crop (Maxted et al., 2006). Plant evolution under domestication has led to increased productivity, simultaneously; domestication has narrowed the genetic variability of crop species so called "founder effect" (Ladizinsky, 1985). As the understanding of agricultural system breeders desires all good traits in one variety which a real challenges for plant breeders, taking into consideration that the new varieties should be higher yielding, nutritious and environmentally sustainable that meets our food demands without harming natural habitats to agricultural production (Zamir, 2001).

It is much needed to exploit the wild ancestors of crop plants to regain the genetic variation that has been lost during domestication. Alien germplasm resources, which include wild species and landraces, often carry many agriculturally undesirable alleles. However, genetic studies can identify the agriculturally valuable traits of wild species, and introgression breeding can transfer these traits to commercial varieties. Wild relatives of cultivated wheat in the tribe *Triticeae* have potential pools of genes for superior traits, which can be transferred into bread wheat and durum wheat through wide hybridization. This potential diversity is distributed within three gene pools; primary, secondary, tertiary; and the exploitation of these alleles makes it possible to enhance the genetic potential of common wheat (Jiang et al., 1994). The gene pool goes much further, for example, bacterial (Golden Rice; Potrykus, 2001) and synthetic genes and increasingly genome-edited genes (Cibus; www.cibus.com) can also be used.

1.5.1 Primary gene pool

The primary gene pool of common wheat (*Triticum aestivum*, 2*n*=6*x*=42, AABBDD) include hexaploid landraces, early domesticates (e.g. *Triticum turgidum*, AABB; *T. monococcum* AA) and wild diploid donors of the A and D genomes [*T. urartu* (AA) and *T. tauschii* (DD)], which are easily crossable to common wheat, hybrids are generally fertile with good chromosome pairings and the gene segregation is normal (Harlan and de Wet, 1971). Transfer of genes from this gene pool is easy and could be achieved via

hybridization, homologous chromosome recombination, backcrossing and selection (Feuillet et al. 2008).

1.5.2 Secondary gene pool

Wheat has a very large secondary gene pool (over 35 species). The secondary gene pool consists of closely related, polyploid species that share at least one homologous genome in common but limited recombination rates with wheat. This includes *Triticum* and *Aegilops* species like *T. timopheevii* (AAGG). The diploid S-genome *Aegilops* species (related to the B genome) in the *Sitopsis* section are also included in the secondary pool because of the reduced chromosome pairing and difficulties in achieving gene transfer. Direct crosses and selection can also transfer genes from the secondary pool if they are located in a homologous genome. Special cytogenetic manipulations are required if they are present in a non-homologous genome (Feuillet et al., 2008).

1.5.3 Tertiary gene pool

Distantly related diploid and polyploid species whose genomes are non-homologous to wheat (other than A, B and D genome) included in the tertiary gene pool. Therefore, homologous recombination cannot usually happen, still, the genomes of species in the tertiary pool are genetically related (homoeologous) to the genomes of wheat, and successful transfer is possible using special cytogenetic manipulation techniques (e.g. irradiation, gametocidal chromosomes, embryo rescue via tissue culture; and often bridge crosses such as VPM1 where *Ae. ventricosa* with *Triticum persicum* (AABB) to obtain fertile amphidiploid and then this wheat was crossed with a common wheat, Marne, to develop VPM1 (= Ventricosa x Persicum x Marne) (Huguet-Robert et al., 2001). Even though such transfers may include an entire chromosome arm or part of an arm, they have been successfully bred into commercial wheat cultivars because the alien chromosome arm or segment genetically compensates for the missing wheat chromatin. Jiang et al. (1994) have reviewed different techniques for gene transfer from this gene pool. This group contains most members of the *Triticeae* that are not within the primary or secondary gene pools. Large proportions in this group are annual (e.g. rye) and perennial (e.g., *Thinopyrum* spp) (Feuillet et al., 2008).

Introgression of alien genes from this gene pool could increase genetic diversity for the agronomic performance of cultivated wheat (Able and Langridge, 2006). The gene

pools of wheat-rye and barley overlap at the tertiary level (Harlan and de Wet, 1971). Hybrids between primary and tertiary gene pool species almost always require in vitro techniques for F_1 rescue due to lethality and physiological abnormality of complete sterility. Embryo rescue is very useful in many combinations but not always necessary, e.g. there is no need for embryo rescue in wheat × rye, and in many wheat × *Aegilops* crosses. High seed set and good germination was achieved in wheat × *Thinopyrum* crosses in Martonvásár without embryo rescue. Embryo rescue is needed for instance for development of wheat × barley hybrids. In these cases, bridging species are often needed to effect gene transfer from the tertiary gene pool to the cultivated crop; Kang et al. (2012) believed the trigeneric hybrid (*Triticum* x *Psathyrostachys* x *Thinopyrum*) could be a useful bridge for transferring *P. huashanica* and *Th. intermedium* chromosomes to common wheat. But this is usually a laborious process, and selection for the desired gene can be very difficult. Many researchers have used tertiary gene pool as a source of resistance genes (biotic and abiotic stress) and have transferred these genes to wheat (see table 1.1).

Table 1.1: List of Wheat-*Thinopyrum* translocations. (Genome symbols 'Ae' or 'Ag' =*Th. elongatum,* genome symbols 'Ai' or 'E'= *Th. intermedium*).

Diseas/ pest	**Gene**	**Translocation**	**Source**	**References**
Leaf rust (*P. recondita f. sp. tritici*)	*Lr19*	7DL-7eL 7D-7Ag#1 T7DS.7DL-7Ae# 1L	*Th. ponticum*	(Sarma and Knott, 1966, Knott, 1968, Dvořák and Knott, 1977)
	Lr24	3DL-3AeL 3D-3Ag1 T1BL.1BS-3AeL	*Th. ponticum*	(Knott, 1968, Sarma and Knott, 1966)
	Lr29	T7DL-7Ae#1L.7Ae#1S	*Th. elongatum*	(Sears, 1973, Sears, 1977)
	Lr38	T2AS.2AL-7Ai#2L T5AL.5AS- 7Ai#2L T1DS.1DL-7Ai#2L T3DL.3DS-7Ai#2L T6DS.6DL-7Ai#2L	*Th. intermedium*	(Wienhues-Ohlendorf, 1960, Wienhues, 1966, Wienhues, 1971, Wienhues et al., 1973, Wienhues,

				1979)
Stem rust (*P. graminis f. sp. tritici*)	*Sr24*	3DL-3AeL 3D-3Ag#1 T1BL.1BS-3AeL	*Th. elongatum*	(Sears 1973; 1977)
	Sr25	7DL-7AeL 7D-7Ag No.1 7A/7AeL No.12 T7DS.7DL-7AeL T6AS.6AL-6AeL	*Th. elongatum*	(Sarma and Knott, 1966, Knott, 1968, Dvořák and Knott, 1977)
	Sr26	T6AS.6AL-6Ae# 1L	*Th. elongatum*	(Knott, 1961, Knott, 1968)
	Sr43	T7DL-7Ae#2L-7Ae#2S T7DS.7DL-7Ae#2L T7DS-7Ae#2L	*Th. elongatum*	(Kim et al., 1992, Kibirige-Sebunya and Knott, 1983)
	Sr44	T7DS-7Ai#1L.7Ai#1S	*Th. intermedium*	(Liu et al., 2013) (Friebe et al., 1996)
Powdery mildew	*Pm40*	7BS	*Th. intermedium*	(Luo et al., 2009)
	Pm43	2DL	*Th. intermedium*	(He et al., 2009)
Barley yellow dwarf	*Bdv2*	T7DS-7Ai#1S.7Ai#1L T1BS- 7Ai#1S.7Ai#1L T7DS.7DL-7Ai#1L	*Th. intermedium*	(Hohmann et al., 1996, Banks et al., 1995)
	Bdv3	7DS.7DL-7EL	*Th. intermedium*	(Kong et al., 2009, Crasta et al., 2000, Ohm et al., 2005)
Wheat streak mosaic	*Wsm1*	T4DL.4DS- 1Ae#1L T6AS-4Ai#2L T6AL.4Ai#2S T4DL.4Ai#2S	*Th. elongatum* *Th. intermedium*	(Sebesta and Bellingham, 1963, Sebesta et al., 1972)
Wheat curl mite (*Eriophyes tulipae*)	*Cmc2*	T5BL.6Ae#2S T6AS.6Ae#2S T6DL.6Ae#2S	*Th. elongatum*	(Larson and Atkinson, 1970, 1972, Larson et al., 1973)

1.6 The *Thinopyrum* genus

Thinopyrum (from Greek: thyno=shore, pyros= wheat) a genus containing 20 species (Dewey, 1984) indigenous to Europe, the Middle East, Central Asia and North Africa is a new genus in the tribe *Triticeae* described by Löve in 1980. Recently Sepsi (2010) has effectively reviewed *Thinopyrum* genus classification. Originally *Thinopyrum* was classified in the *Agropyron* complex based on morphological characteristics (having one spikelet per node), which included almost all the perennials making it a very large, diverse genus having more than 100 species (Hitchcock, 1951). This definition of the genus was not specific and rather confusing. Later on, the former Soviet Union taxonomist Nevski (1933) applied a cytological method along with morphology to divide Agropyron into four relatively homogeneous genera: *Agropyron*, *Roegneria*, *Antosachne* and *Eremopyrum*. He narrowed down the *Agropyron* genus into a dozen species closely related to crested wheatgrass (*A. cristatum*). Further Löve (1980 partitioned the *Agropyron* genus into genera: *Agropyrum*, *Pseudoroegneria*, *Thinopyrum*, *Trichopyrum* and *Lophopyrum. Agropyrum* was limited to less than 10 species, including the type species *A. cristatum* (2n=14, PP), whose genome was designated as P. All species that contained other than the P genome were excluded from *Agropyron*. Löve (1982, 1984) established the *Thinopyrum* genera on the basis of their genomic constitution. *Thinopyrum* was formed as a new genus to include species carrying the J-genome and *Lophopyrum* for species carrying the E-genome. Thus, J and E genomes were assigned to these well-defined genera. Dewey (1984) suggested that J genome of *Thinopyrum* and the E genome of *Lophopyrum* are closely related and should be combined into one genus while retaining the J genome designation. Löve considered *Lophopyrum* as a distinct genus, with the genomic designation E, while Dewey divided the *Thinopyrum* genus into three sections (*Thinopyrum, Lophopyrum, Trichophorae,*). Löve initially established *Thinopyrum* genus with only six species carrying the J genome composition *Thinopyrum junceum* complex. They all are maritime grasses growing on the shore. Dewey expanded *Thinopyrum* to about 20 species, including three species from the genera *Lophopyrum* and *Elytrigia* (Dewey 1984).

1.6.1 Thinopyrum bessarabicum

Thinopyrum bessarabicum (2*n*=2x=14, E^bE^b=JJ) is a perennial; rhizomatous seashore wheatgrass distributed in the Black Sea and Mediterranean region. It possesses salinity tolerance and resistance to several diseases, and can be a significant gene source for wheat improvement (Gorham et al., 1985, William and Mujeeb-Kazi, 1993, King et al., 1997).

1.6.2 Thinopyrum intermedium

The intermediate wheatgrass *Thinopyrum intermedium* (2*n*=6x=42) is a segmental auto-allohexaploid, which has two closely related, partially homeologous genomes and one distinctly diverse genome (Dewey 1984). Several studies have been done to identify and understand gnome constitution of *Th. intermedium;* the recent studies indicated that it has three distinguishable chromosome sets designated as J, J^S, and St genomes. The J genome was related to both *Th. elongatum* and *Th. bessarabicum*; however, the J^S genome referred to a modified *Th. elongatum*/*Th. bessarabicum* genome (Chen et al., 1998, Chen, 2005). This is the perhaps most important grass exploited by breeders among Thinopyrum genus. Several wheat-*Th. intermedium* amphiploid and addition lines have been developed. The partial amphiploid, MT-2, is a valuable perennial wheat line derived from hybridization between durum wheat and *Th. intermedium* (Schulz-Schaeffer and Haller, 1987).

1.6.3 Thinopyrum ponticum

The most important species among *Thinopyrum* genus is *Thinopyrum ponticum,* a decaploid species (2*n*=10*x*=70), often used in wheat improvement as a donor of various disease resistance genes, in particular for leaf rust and wheat streak mosaic virus (Sebesta et al., 1972, Martin et al., 1976, Jiang et al., 1993, Friebe et al., 1996). The genomic constitution of *Th. ponticum* is still undetermined, efforts have been underway for decades and various hypotheses have been proposed. In early days, Cauderon (1966) and Muramatsu (1990) studied chromosome pairing behavior during meiosis, they suggested that *Th. ponticum* is an autopolyploid species and its genomic formula was designated as $J_1 J_2 J_3 J_4 J_5$. Later on Zhang et al. (1996) using GISH demonstrated that E^b (=J genome) from *Th. bessarabicum* and S^t genome from *Pseudoroegneria spicata* were actually the two basic genomes of *Th. ponticum.* Subsequently Chen et al. (1998) using

S genomic DNA as a probe revealed that the genomic composition of *Th. ponticum* was J^sJ^sJJJ. The J genome is homologous to the J genome of the diploid *Thinopyrum bessarabicum*, whereas the J^s genome is a modified J genome of unknown origin characterized by the presence of an S genome-specific hybridization signal near the centromere. The wheat–*Thinopyrum ponticum* partial amphiploid, BE-1 ($2n=8x=56$), was produced by Szalay (1979), having high protein content and resistance to leaf rust and powdery mildew and was characterized by Sepsi et al. (2008) using GISH and FISH.

1.7 Chromosome engineering in wheat

Chromosome engineering has been an integral part of wheat cytogenetics following Sears' classic experiments on alien gene transfers using x-ray irradiation and induced homoeologous pairing (Sears 1956, 1972, 1973). Chromosome engineering describes the technologies, oriented on manipulating chromosomes in order to change the inheritance of genetic traits (Chan, 2010, Pershina, 2014). The concept of "chromosome engineering" was introduced by the American researcher E. Sears in 1972, based on summarizing the results of his studies on the transfer of chromosome segment of *Aegilops umbellulata* in the genome of common wheat, carried out in 1956. The term chromosome engineering includes activities like to induce inversions, translocations, and rearrangements. However, in this thesis, the term is used to denote the transfer of alien segments. According to E. Sears, the ultimate aim of chromosome engineering is to targeted transfer of alien chromosome segments into the genome of cultivated plants from other species in order to enhance variability, and thus, increase the efficiency of plant breeding improve crop traits. It has been developed to overcome linkage drag and by reducing the size of the alien chromosome segment transferred to a crop plant genome.

Several methods of chromosome engineering have been described in wheat. These methods can be categorized based on when they were first used; whether whole chromosome arm or small segments are involved; whether random chromosome breaks or directed translocations are involved. The four methods, listed consecutively, are using ionizing radiation, induction of homoeologous pairing, spontaneous induction, and use of gametocidal chromosome (Cainong, 2014).

1.7.1 Using ionizing radiation

Sears (1956) developed this method using ionizing radiation to produce translocations between a wheat chromosome and an alien chromosome from *Ae. umbellulata* (2*n* = 2x =14; UU) carrying *Lr9*, a gene for resistance to leaf rust *Puccinia recondita* f. sp. *Tritici*, to chromosome arm 6BL. This particular translocation is the only one intercalary translocation out of the forty-translocation lines had produced. Several other genes were similarly transferred, but the most successful transfer involved the transfer of a portion of *Agropyron elongatum* chromosome 6el carrying stem rust resistance gene *Sr26* to chromosome arm 6A (see Knott, 1971 for review). Wheat genetic stocks such as amphiploid, addition line, and substitution line of wheat–alien species were usually used as initial materials to induce alien chromosome translocations. Ionizing radiation breaks chromosomes at random and fusion of broken segments results in translocated chromosome and it could be different from the original chromosome. The segments transferred are usually smaller than whole chromosome arms. However, the procedure is laborious and the translocations are random, most are non-compensating types and have deleterious effects. A chemical agent such as Ethyl Methanesulfonate (EMS) has also been used to induce chromosome breakage. Among the most important radiation-induced mutations in the Triticeae is the dwarfing gene in barley, now present in many if not most modern barley varieties for example 'Diamant' and 'Golden Promise', this two mutant varieties have had a major impact on the brewing industry in Europe (IAEA Mutant Variety database). Molnar et al. (2009) detected irradiation-induced translocations in wheat-*Aegilops biuncialis* amphiploids among the U and M chromosomes of *Ae. biuncialis* and chromosomes of hexaploid wheat.

1.7.2 Induction of homoeologous pairing

After observing number of trivalents and some quadrivalents (in contrast to bivalents only in diploid wheat) in the wheat stocks lacking for chromosome V (later designated as chromosome 5B), Okamoto (1957) and Riley and Chapman (1958) indicated that the gene which allowed pairing between homologous chromosomes only was located on this chromosome. Soon after, Riley (1960) demonstrated that the controlling gene was present on the long arm of this chromosome. Wall et al. (1971) first called this dominant gene, regulating the chromosome pairing in wheat is now known as *Ph1 (pairing homoeologous)*.

This second method of chromosome engineering has more accuracy and control than the previous method. This is because it emphases on the homoeologous relationship between alien and wheat chromosomes. There are three different ways to induce homoeologous pairing. First is by suppressing the effect of *Ph1* gene (Riley et al., 1968a; 1968b), second is by eliminating chromosome 5B (Sears 1972), and third is by using the *ph1b* mutant (Sears 1981).

Riley and co-workers (1968a; 1968b), pioneers of homoeologous pairing by suppressing the effect of *Ph1* gene, used a high pairing accession of *Ae. speltoides* to induce recombination between wheat and *Ae. comosa* chromosomes to transfer stripe rust and stem rust resistance genes (*Yr8* and *Sr34*) to wheat. Qi et al. (2007) reviewed a method to induce homoeologous pairing with the use of *ph1b* mutant.

In this method a translocation line containing an alien chromosome is crossed to a homozygous *ph1b* stock. The resulting hybrid is further backcrossed to the mutant *ph1b* stock to obtain a plant that is homozygous for *ph1b* and heterozygous for the translocation. Selfing of this backcrossed hybrid will then recover recombinant chromosomes. Homoeologous recombination techniques can be used to reduce the size of the alien segment including the target gene(s) incorporated into the wheat chromosome. The selection of such recombinants is a critical step, which occur only at low frequency (King et al., 1993, Qi et al., 2007).

However use of homoeologous chromosome pairing has also its own limitations as the recombination between homoeologous chromosomes of wheat and related species is either absent or drastically reduced in the proximal regions of chromosome arms, making it difficult to transfer a target gene from these regions (Lukaszewski, 1995, Lukaszewski and Curtis, 1993, Werner et al., 1992). Because of this it is always difficult to transfer a target gene from these areas using induced homoeologous recombination. In these cases, radiation treatment with strong selection for the recovery of compensating translocations (Sears 1956) or other methods such as the use of the chromosome breaking action of gametocidal chromosomes (Endo, 2007, Endo et al., 1994) might be more successful. The six wheat-*Th. bessarabicum* translocation lines used in this study were developed using this method.

1.7.3 Spontaneous induction

Sears (1972) proposed this third method for chromosome engineering in wheat, which involves the transfer of whole chromosome arms. This method utilizes the centric-breakage-fusion mechanism of univalents at meiotic metaphase I (Sears, 1950). Sears observed that at the first division of meiosis, univalents misdivide producing either telocentrics or isochromosomes. In plants with 20" + 1'W + 1'A, the univalent usually tend to break at the centromeres. If both misdivide in the same cell, a wheat chromosome arm may rejoin with an alien chromosome arm to form Robertsonian whole arm translocations (Robertson, 1916). However, the frequency is so slow that the method may be unfeasible (Sears, 1981). Friebe et al. (2005) studied how these Robertsonian translocations were formed. They found that at anaphase or telophase of meiosis I, centric misdivision of univalents form telocentrics, which then segregates to the same nucleus and fusion of the broken ends during interkinesis can result in Robertsonian translocations. Zeller and Koller (1981) produced two wheat-rye whole arm translocations using crosses between Chinese Spring monosomics and Chinese Spring – rye addition lines. The best and most popular example of centric misdivision is the 1RS·1BL translocation in which the short arm of chromosome 1 of rye (1RS) transferred to the long arm of chromosome 1B of wheat (1BL). The 1RS·1BL translocation occurs naturally in several breeding programs in Europe and worldwide breeding programs (Zeller and Hsam, 1983, Lukaszewski, 1990).

Sometimes in wide crosses spontaneous translocations occur between alien and wheat chromosomes. For example the old Portuguese wheat landrace 'Barbela' where rye-origin chromosome segments were introgressed spontaneously in the distal region of wheat chromosome arm 2DL. This landrace shows good fertility in acid soils (Ribeiro-Carvalho et al., 2001). Similarly, a spontaneous translocation involving chromosome 3D occurred in the production of leaf and stem rust resistant cultivar Agent from a cross involving a wheat – *Ag. elongatum* derivative (Smith et al., 1968). Resistance derived from Agent has been used in a number of USA cultivars. Several European wheat cultivars carry a spontaneous 1B/1R (T1BL.1RS) translocation (Mettin et al., 1973, Zeller et al., 1973). Zhou et al. (1997) found *P. juncea* chromosome segments either translocated to wheat chromosomes or as telosomes in *Triticum aestivum x Psathyrostachys juncea* hybrids.

1.7.4 Use of gametocidal chromosomes (Gc)

In wheat gametocidal chromosomes (Gc) can induce random chromosome breaks and rejoins in gametes without the Gc gene during the first division of pollen mitosis (Nasuda et al., 1998). The breakage occurs at random in both the alien and the wheat chromosomes resulting in non-compensating translocation and deletions (Endo, 1988, Endo, 2007). The gametocidal chromosomes (Gc) were first detected in the *Aegilops* species. The use of *Gc* is a unique genetic system to produce genetic stocks with terminal deletions of various sizes. The broken chromosome ends, if not fused to other ends, are healed by the addition of telomeric sequences making up the telomere (Werner et al., 1992). The action of *Gc* segments results in translocation chromosomes. The *Gc* system is an effective way to produce wheat-alien translocations; it causes chromosome breaks in alien chromosomes that are added. It is a way to transfer genes located in the proximal regions, which in normally difficult to transferred by induced homoeologous recombination (Cainong, 2014). The *Gc* system has been used to develop barley-wheat translocation lines, which have been derived from hybrids multiplied in vitro (Mólnar-Láng et al., 2000; Endo et al. 1998).

1.8 *In situ* hybridization (FISH) and (GISH)

Gall and Pardue (1969) and John et al. (1969) performed first DNA *in situ* hybridization. *In situ* hybridization involves the use of probes (originally with radioactive nucleotides, and now mostly other labels detected by fluorescence) to detect the specific DNA sequences. As an important molecular cytogenetic technique, FISH has been widely applied to plant genome research and molecular breeding in recent years. Jiang and Gill (2006), Younis et al. (2015) has extensively reviewed FISH and its importance. It is being widely used for identification and physical mapping of DNA sequences within the genome, which can then be precisely used to measure the distance between the various genes or repetitive elements (Yang et al., 2011). It is also useful for correlating linkage groups to specific chromosomes, and for understanding genome organization and the three-dimensional spatial distribution of DNA sequences at interphase and meiosis (Leitch and Heslop-Harrison, 1993, Schwarzacher, 2003). FISH is an excellent way to study chromosome polymorphism and karyotyping (Linc et al., 2011, Sepsi et al., 2008). FISH pattern generated by repetitive DNA probes produce a distinctive and stable karyotype for each species (see Fig. 1.2, 3.39). FISH is very

helpful for studying copy numbers at various locations, the DNA sequence distribution on the chromosome, and for observing the evolutionary variations to their physical structure in the genome (Harrison and Heslop-Harrison, 1995). *In situ* hybridization is also an efficient method for the physical mapping of transgenes in transgenic plants (Salvo-Garrido et al., 2001). It has also been used to study the distribution of repetitive sequences in the genome (Cuadrado and Schwarzacher, 1998, Cuadrado et al., 2000, Cuadrado et al., 2008).

The method of using genomic DNA cloned in large-insert vector bacterial artificial chromosome (BAC) as probe during fluorescence *in* situ hybridization (FISH) experiment is called BAC-FISH. This has been successfully used in the plants with small genomes also in wheat, which has a large genome. It is a useful method for identifying molecular cytogenetic markers to determine physical location of specific DNA sequences and chromosome identification (Dong et al., 2000, Jackson et al., 2000, Jiang et al., 1995, Fransz et al., 2000, Zhang et al., 2004a, 2004b). This can help trace and identify any specific chromosome section or an entire chromosome during the meiotic stage, and this also enables structural chromosome rearrangements and meiotic-pairing to occur (Lysak and Manda´kova´ 2013). BAC-FISH is now the most common way of detecting low copy and individual DNA sequences, and gene locations on chromosomes.

Similarly, genomic *in situ* hybridization involves the use of total genomic DNA as probes labelled with fluorescent labels, and detected at specific frequencies, hybridized to the chromosomes of hybrid. GISH has successfully used in the identification of parental donors of different hybrids, alien donors in alien hybrids and amphiploid, (Schwarzacher et al., 1989, Sepsi et al., 2008). GISH has also been used successfully for the identification of translocation, deletions and alien introgressions (Ribeiro-Carvalho et al., 2001, Cai et al., 1998, Molnár-Láng et al., 2000).

1.9 Objectives

General objective

The overall aim of the project is to identify and provide genetically and physically characterized wheat-*Th. bessarabicum* lines with recombinant chromosomes, along with a structured set of tools (advance cytogenetics and next generation sequencing) that will enhance and encourage the exploitation of the lines by breeders.

Specific objective

1. To identify and characterize size and chromosomal origin of *Thinopyrum* chromatin segments and the segment size and linkage group of the recipient wheat chromosome using molecular cytogenetic tools in public lines from international programs.
2. To identify and karyotype *Th. bessarabicum* chromosome ($2n=2x=14$, JJ genome).
3. To define the wheat alien breakpoint and identify the DNA sequences and genes those are involved, with cytology and guided molecular markers.
4. To map wheat-*Th. bessarabicum* translocations using SNPs generated by Genotyping by Sequencing (GBS).
5. To cross and backcross alien and recombinant wheat lines. Substantial improvements in molecular and cytological methods over the last decade mean we can apply it to substantial numbers of lines, with the quick characterization of alien segments.

Chapter 2: Materials and methods

2.1 Plant Materials

2.1.1 Wheat-Thinopyrum hybrids

Six *Thinopyrum bessarabicum* translocation lines had been produced in CIMMYT, Mexico, in a Prinia (CIMMYT bread wheat) background, and initially selected for salt resistance. These lines were developed by manipulation of the *Ph1* genetic control mechanism (Sears 1977) involving Professors Mujeeb Kazi, Sanjay Rajaram, and Adam Lukaszewski in the CIMMYT wide-crossing long-term research program. Seeds were obtained from Dr Masahiro Kishii (CIMMYT, Mexico) and the detailed pedigree of lines is given in table 3.1 in Chapter 3.

All the lines included in this study are often referred to as a wheat-*Th. bessarabicum* hybrid or introgression or recombinant lines. They were germinated in Petri dishes and grown in the growth cabinet facility under controlled conditions.

2.1.2 Seed germination and multiplication

Seeds were germinated in dark on filter paper moistened with mineral water in Petri dishes for 3 days until roots were 1-2 cm long. Seedling root tips were collected and pretreated for *in situ* hybridization experiment. After collection of root tips, seedling were transferred to Jiffy pellets (LBS horticulture Ltd). After they had grown, seedlings were transferred to a growth cabinet facility with 25^0C temperature and 16 hrs daylight.

2.2 Methods

2.2.1 Isolation of total genomic DNA

The total genomic DNA was extracted from young leaves using the modified CTAB method (Doyle, 1990). In brief, 2-4 young and healthy leaves were collected from individual plants and washed with distilled water. For extraction about 1g of the young leaves was wrapped in silver foil. This was then kept in liquid nitrogen and then immediately ground to a fine powder using mortar and pestle to prevent enzymatic degradation. Roughly about half a spatula of PVP (Polyvinylpyrolidone, Sigma) was added to this fine powder. 5 ml of CTAB along with 20 µl mercaptoethanol was

preheated in 50ml Falcon tubes, and the fine powdered leaf was added to this. Tubes were incubated at 60°C for 30–60 min in a shaking water bath. To this, an equal volume of chloroform–isoamyl alcohol (24:1) was added and mixed repeatedly by inverting the tubes for 3 min followed by centrifugation at 5000 rpm at RT for 10 min. The supernatant was then carefully transferred to a new Falcon tube. The chloroform–isoamyl alcohol step was repeated once more and the supernatant transferred to a new tube. The DNA was then precipitated with 0.6 volume of pre-chilled isopropanol mixed gently by inverting and then kept on ice for 10 min. Precipitated DNA was spooled out with a sterile glass rod or spun down at 3000 rpm for 3 min (in case there wasn't much to spool out), dried and washed with 5 ml of wash buffer for 20 min. The DNA was finally air dried and re-suspended in 1x TE buffer and kept at RT overnight. RNase A (2 µl of 10 mg/ml) (Bioline) was then added to this DNA and incubated at 37°C for 1 h, to get rid of any RNA. The DNA was then re-precipitated with the 1x volume of sodium acetate 3M (pH6.8) and 2x volume of pre-chilled absolute ethanol. The DNA was then spun down and re-suspended in 500 µl of 1x TE buffer and left overnight. The DNA was finally stored in -20ºC freezer. DNA was allowed to thaw on ice before use.

2.2.2 DNA Quantification

DNA quantification was done using the Thermo Scientific NanoDrop 8000 Spectrophotometer with full-spectrum wavelength ranging from 220-750nm. The sampling arm was opened and set to blank using 1 µl of sterile water. 1 µl of the sample was then directly dispensed onto the lower measurement pedestal and the sampling arm was closed and the spectral measurement was initiated using the software. The readings were taken at 230nm, 260nm and 280 nm; 260nm absorbance was for concentration measurement and the ratios were used to check quality.

2.2.3 Agarose gel electrophoresis

The genomic DNA was separated using agarose gel electrophoresis. Agarose gels (0.8-2%) were prepared by boiling the agarose in a microwave oven (Melford and Bioline, molecular grade) in 1x TAE. This was cooled down to 45°C (hand touch) before adding ethidium bromide to a final concentration of 0.5 µg/ml carefully in fumehood. Gel combs were placed in sealed gel trays to make wells, and the agarose was poured into this and allowed to settle down. The DNA samples were mixed with appropriate amounts of 6x loading buffer (sometimes dilutions of 3x were used). These were then

loaded in the wells along with the DNA marking ladder, hyperladder I (Bioline) with known band DNA concentrations. This was allowed to run from about 45-60 min at 7V/cm and visualized with GeneFlash (Syngene) gel documentation system.

2.2.4 SSR markers

Appropriate PCR markers were chosen from international databases. Total 75 wheat microsatellite markers were selected from the GrainGenes 2.0 database (http://wheat.pw.usda.gov/GG2/index.shtml; Xgwm: Röder et al., 1998a, 1998b; Xbarc, Xwmc: Somers et al., 2004; Xgdm: Pestsova et al., 2000; Xcfa, Xcfd: Sourdille et al., 2004). List of SSR markers along with annealing temperature is given in Appendix 1.

2.2.5 PCR

Amplification was done by PCR using Tprofessional Gradient Thermocycler (Biometra) in a 15 µl reaction mixture containing 50 ng of template DNA. The reaction mixture contained Kapa biosystems bufferA (1x; 750 mM Tris-HCL pH 8.8, 200mM $(NH_4)_2SO_4$, 15mM $MgCl_2$, 200 µM of dNTPs (Bioline), 0.6 µM of each primer, and 0.5 U of Kapa Taq DNA polymerase.

The standard PCR conditions were 94ºC for 4 min followed by 40 cycles of 94ºC for 1 min, 55-64ºC (depending on annealing temperature of different primer sets) for 45 s, 72ºC for 2 min, and final extension of 72ºC for 7 min followed by holding the block at 16ºC. The PCR product was then accessed by electrophoresis mentioned above on agarose gel (2.5%). The images were taken in the Geneflash and labelled in Photoshop.

2.3 Probes used

FISH was carried out using the following repetitive sequences:

- ***pTa535*:** contains a 342 bp tandemly repeated DNA isolated from Chinese Spring (*Triticum aestivum*) (Komuro et al., 2013).
- ***pSc119.2***: contain a 120 bp tandemly repeated DNA sequence isolated from *Secale cereale* (McIntyre et al., 1990).
- ***pTa71***: contains a 9 kb *Eco*RI fragment of the repeat unit of 25S-5.8S-18S rDNA isolated from *T. aestivum* (Bedbrook et al., 1980).

- ***dpTa1***: containing tandem repeat with a monomeric length of 340bp isolated from *T. aestivum* was subcloned by Vershinin *et al*., (1994) and is homologous to *pAs1* (Rayburn & Gill 1886), *pHcKB6* from *Hordeum chilense* (Anamthawat-Jónsson & Heslop-Harrison 1993) and the *Afa* family (Nagaki et al., 1995; Nagaki et al., 1998).
- ***pTa794*:** contains a 410 bp fragment of 5S rDNA of *T. aestivum* (Gerlach & Dyer 1980).
- **GAA microsatellite:** GAA satellite sequences were amplified from the genomic DNA of *Hordeum vulgare* and labelled with biotin-16-dUTP using PCR (Vrána et al., 2000).
- **Genomic DNA probes** Genomic DNA from the species (*Th. bessarabicum* and *T. monococcum*) was first sheared in an autoclave at 110°C for 3 min were labelled by random priming.

2.4 Probes Labelling

2.4.1 PCR labelling

DNA clones less than 500 bp in size was labelled with PCR using universal M13 primers with reactions including 1.8 µl of biotin-16-dUTP or digoxigenin-11-dUTP (1 mM, Roche Diagnostics). 1µl of water was used as a control for this PCR set up. The PCR conditions described above.

2.4.2 Random primers labelling

Total genomic DNA and the clones larger than 500 bp in size were labelled with a random primer labelling kits (Bioprime DNA labelling system, Invitrogen). The DNA was denatured and the single stranded DNA is amplified using a random mixture of oligonucleotides using DNA Polymerase I (Klenow fragment) of *E.coli* using kits from Invitrogen.

Genomic DNA was sheared to 3-5 kb pieces by autoclaving at 110°C for 3 min before labelling. The fragment size was estimated by running the autoclaved DNA on 1% agarose gel. Probes between 500 bp -2 kb were labelled with BioPrime® DNA labelling System (Cat.No.18094-011) for biotin, and random primer DNA labelling system (Cat.No.18187-013) for digoxigenin incorporation. Genomic DNA and large clones of

several kb were labelled with BioPrime® Array CGH Labelling System (Cat.No.18095-011). Labelling reactions were performed in a final volume of 50 µl, following manufacturer's instructions. Labelling was achieved with 200 ng of the purified cloned DNA or 500 ng - 1 µg of sheared genomic DNA mixed with 20 µl of 2.5x Random primer solution, denatured in boiling water for 5 min to open up the DNA strands, and then chilled on ice for 5 min. To this mixture 5 µl of 10x dNTP mix and 1 µl of 40U Klenow fragment was added and incubated at 37ºC for 1 h for biotin labeling with BioPrime®DNA Labelling system. For digoxigenin labelling with Random Primer DNA Labelling system, 2 µl of dATP, dCTP, dGTP and 1 µl of TTP together with 1 µl of digoxigenin-11-dUTP (1mM) and 2 µl of Klenow fragment (3U) were mixed with the denatured DNA mixture and incubated at RT overnight. Labelling reactions with BioPrime® Array CGH Genomic Labelling System involved the addition of 3 µl of biotin-16-dUTP or digoxigenin-11-dUTP (1mM), 3 µl of 10x dUTP nucleotide mix and 1 µl of exo-Klenow fragment (40U) to the denatured DNA mixture. The reaction was incubated at 37ºC for 2 h. The reactions were stopped after the incubation period by adding 5 µl of manufacturers stop buffer (0.5 M EDTA pH 8.0). Labelled probes were purified to remove any unincorporated nucleotides, enzyme and salts using NucleoSpin® Extract II Kit (Macherey Nagel), following manufacturer's instructions.

2.5 Testing of labelled probes (dot blot test)

Colorimetric dot blot test was conducted to check the incorporation of the labelled nucleotides the strength of incorporation. A positively charged nylon membrane (Hybond N+ from Amersham Biosciences) of appropriate size depending upon the number of probes was cut and marked with a pencil at the edge for identification later. This membrane was soaked in buffer 1 for 5 min and then blot dried between filter paper. Small spots of labelled DNA (0.5-1 ml) were micro-pipetted, along with the controls of previously tested DNA, onto the membrane and left to adsorb and partly dry for 5-10 min with spot identifications on membrane added with a pencil. Place the membrane in a Petri-dish with 4 ml buffer 1 for 1 min, and then place in 4 ml buffer 2 for 30 min. Shake gently, pour off buffer and distribute 0.5 ml, 1:500 in buffer 1 (0.75 U/ml) antibody-AP mixture (anti-biotin-alkaline phosphatase and anti-digoxigenin-alkaline-phosphatase, Roche Diagnostics) over the membrane, cover with a plastic sheet and incubate at 37°C for 30 min, shaking gently from time to time to keep it agitated.

The membrane was then washed in buffer 1 for 15 min. The membrane was then equilibrated in buffer 3 for 2 min and detected with INT/BCIP (Roche Diagnostics). The stock solution of INT/BCIP [33mg/ml 2-(4-iodophenyl)-5-(4-nitrophenyl)-3-phenyltetrazolium chloride and 33 mg/ml 5-bromo-4-chloro-3-indonyl-phosphate, toluidine-salt in DMSO] was diluted 1:500 in buffer 3 and the membrane detected at RT for 10-15 min in dark to develop fully. The membrane was washed with distilled water and allowed to dry. Dark and light brown spots were seen on the membrane due to the colorimetric reaction of the detection reagents. The intensity of the colour was indicative of the labelling efficiency.

For the PCR labelled probes, 1 µl of PCR product was loaded on 1% agarose gel. Successful incorporation was indicated by the retardation of the same sized band in biotin and digoxigenin as compared to the control reaction run parallel to it. These probes were later used in fluorescent *in situ* hybridization.

2.6 Collection and fixation of root tips

Seeds were germinated on filter paper moistened with bottled drinking water in Petri dishes for 3 days until roots were 8 to 12 mm long. Seedling root tips were pretreated ice water for 24 hrs or in at 4°C and then fixed in freshly prepared 3:1 (v/v) ethanol: glacial acetic acid (24 h), and stored at 4°C until use.

2.7 Chromosome preparation

2.7.1 Mitotic spreads

Chromosome preparations were made from the root tips of plants using standard enzymatic digestion techniques (Schwarzacher and Heslop-Harrison, 2000a). In brief, plant root tips were incubated in ice-cold water for 24-26 hours at 4^{o}C temperature prior to fixation in fresh 3:1; ethanol: acetic acid and left overnight. For metaphase chromosome preparation, roots were washed with the enzyme buffer for 5 min and then water for 1 min before digesting in an enzyme solution 0.1 % (w/v) cellulase onozuka RS (Sigma- Aldrich), 0.1 % pectolyase Y23 (Sigma-Aldrich) in 10 mM citrate buffer, pH 4.8 for 90 min at 37°C. After digestion the enzyme solution was replaced with the enzyme buffer, and then a single root tip was squashed in 60% (v/v) acetic acid under a stereomicroscope on a clean glass slide (SuperFrost®, Menzel- Glaser, Thermo

Scientific). The root cap and other permanent tissues were removed by using fine needles and forceps. The squash was covered with a 18mm x 18mm coverslip and thumb pressure was applied. The slide was briefly passed over the flame to dissolve the cytoplasm and make cells flat. Slides with good metaphases and well spread complete chromosome sets (42 chromosomes for wheat) were selected and placed on dry ice for freezing. After freezing, coverslips were removed using a razor blade and the area with cell material was marked with the diamond pen. Slides were then dried and stored at -20°C until hybridization.

2.8 Fluorescent *in situ* hybridization

2.8.1 Pre-hybridization

The slides with high metaphase index were selected and re-fixed in cleaned freshly prepared alcohol: acetic acid fixative for 25 min followed by dehydrating with absolute ethanol (100%) twice for 5 min each. The slides were then air-dried. 200 µl of RNase A (100 µg/ml) diluted in 2x SSC was then added to the marked area of each slide and covered with a large plastic coverslip and incubated for 1 h at 37°C in a humid chamber. The slides were then washed with 2x SSC twice for 5 min each, after removing the coverslips carefully. The slides were then incubated in 0.1 mM HCl for 5 min. Excess solution was taken off and 200 µl of pepsin (stock 1 mg/ml) in a concentration of 5 µg/ml was applied to the slides and covered under plastic coverslip and incubated in 37°C for 20 min in a humid chamber. The coverslips were then removed and to stop the reaction the slides were placed in distilled water for 1 min. Wash slides in 2x SSC twice for 5 min. The slides were then re-fixed in a freshly prepared 4% (w/v) paraformaldehyde at room temperature for 10 min and then washed with 2xSSC twice for 5 min. The slides were then dehydrated in a series of 70%, 85% and 100% ethanol, for 2 min each. The slides were then air-dried in a rack. The slides were checked under the phase contrast microscope to check for the possible loss of cells and the clearing of cytoplasm due to pepsin treatment.

2.8.2 Hybridization

The hybridization mixture (35–40 µl per slide) containing 50% (v/v) deionized formamide, 10% (w/v) dextran sulfate, 0.125 % (w/v) sodium dodecyl sulfate, 1 µg salmon sperm DNA, 0.125 mM EDTA, 2× SSC and 25 -100 ng of probe (Schwarzacher

and Heslop-Harrison, 2000), was applied to each slide. For genomic *in situ* hybridization, a various quantity of blocking DNA, depending upon the species (mentioned with the specific concentration in chapter 3) was also added to the hybridization mixture. The probe mixture was denatured at 80°C for 10 min then cooled on the ice at least for 10 min. The mixture was then placed onto the slides, covered with a plastic coverslip. The probe and preparation were then denatured together at 71°C for 7 min, under a plastic coverslip on a Hybaid Omniblock and in Thermocycler before cooling down slowly to 37°C for overnight hybridization of about 16-20 h with the machine set on vibration. The stringency of the hybridization mixture is dependent on the formamide concentration, the Na^+ in the SSC and the temperature of denaturation. These were varied sometimes for attaining better stringency and specificity of the probes. The blocking DNA and the salmon sperm DNA help reducing the non-specific hybridization while the dextran sulfate increases the volume without diluting the probe. EDTA stops the nucleases while SDS helps for the better penetration of the probe (Schwarzacher and Heslop-Harrison, 2000).

2.8.3 Post hybridization

After the overnight hybridization, the slides were washed to remove the unbound probe and any remaining hybridization mixture. The stringency was varied in different experiments to attain maximum specificity. Slides were placed in a Coplin jar and the coverslips were floated off by incubating the slides in 2xSSC at 42°C. The coverslips were taken out with the help of forceps. For low stringency, slides were washed with 0.01x SSC twice for 5 min and 10 min respectively, followed by a wash with 2x SSC for 5 min at room temperature. For a high stringency, the stringent wash step was exchanged with two washes of 2x formamide (25%) followed by one wash with 0.1x SSC and then slide were cooled at room temperature in 2x SSC for 5 min.

2.9 Slide detection

For the detection of the probes, slides were incubated in detection buffer for 5 min at room temperature. This was followed by the addition of 200 µl of 5% BSA (Bovine Serum Albumin), made in detection buffer and incubated in a humid chamber at 37 °C for 30 min. For the detection of hybridization sites 40 - 50 µl of 2 µg/ml streptavidin conjugated (1 mg/ml stock, Sigma) to Alexa594 (Molecular probes) and 4 µg/ml FITC-

anti-digoxigenin (fluorescein isothiocyanate, 200 mg/ml stock, Roche Diagnostics) was made in the blocking solution (5% BSA). Slides were incubated in a humid chamber for 1 h, followed by two washes with the detection buffer at 42 °C to remove the extra antibodies.

2.10 Slide mounting

The slides were counterstained with 80 µl of 4 µg/ml DAPI) diluted in McIlvaine's buffer for 25 min in the dark. The slides were rinsed in detection buffer before mounting in a drop of anti-fade solution (Citifluor, Agar Scientific) under a No. 0 24 mm x 40 mm coverslip. The slides were stored at 4°C overnight to stabilize the fluorescence achieved by the binding of the antifade to the fluorophores.

2.11 Photography

Slides were examined with an epifluorescence Zeiss Axiophot microscope and images were captured with a ProgRes C12 cooled CCD camera (ProgRes ^{TM} C12, Optronics, model S97790) and Nikon 80i. For the Zeiss microscope the digoxigenin probe was analysed using filter set 10 (excitation= BP450-490, beam splitter=FT510 and emission= BP515-565), and detected with antidig FITC. Filter set 15 (excitation= BP546/12-490, beam splitter=FT580 and emission= LP 590) for biotin labelled probes, and was detected with streptavidin FITC, whereas the DAPI stained chromosomes were analysed with UV band pass filter (filter set 01, excitation = BP365/12, beam splitter =FT395 and emission = LP 397). For Nikon80i microscope the camera used, was DS-QI1Mc-U2 12 bit, with a numerical aperture of 1.3 and the refractive index 1.515. The emission and excitation of the red, green and DAPI fluorescein were 620 and 540.5 respectively with the channel modality being a wide field. Each metaphase was captured in three different channels and over layered using Adobe Photoshop CC using only functions including contrast and brightness adjustment that affect the whole area of the image equally.

2.12 Reprobing of slides

The slides used for FISH, GISH can be re-probed to see multiple probe signals on the same cell. For reprobing different protocol were followed based on chromosome morphology (Komuro et al., 2013, Schwarzacher and Heslop-Harrison, 2000, Heslop-Harrison et al., 1992). Firstly the immersion oil was removed from the surface of the coverslip carefully. The slides were then placed in 37 °C for 5 to 10 min to reduce the viscosity of the glycerol/antifade mountant. The coverslips were then removed by lifting slowly but steadily with the edge of a razor blade. The slides were washed in a Coplin jar with the detection buffer for 5 min at room temperature.

Wash slides in a staining jar with detection buffer at room temperature for 5 min and then twice for 30-60 min. This is followed by incubating the slides in 2xSSC for 2 times 5 min at room temperature. The slides were then dehydrated with 70%, 85% and 100% ethanol and air-dried. Hybridization, washing and detection were same as above.

After capturing images, the coverslip was removed, and the slide was washed gently with 70% ethanol. The slide was sub- merged in a boiling 2× SSC buffer (100°C) for 5 min to remove probes. The slide was washed with distilled water and then rinsed with 70% ethanol briefly and air-dried. The dried slide was examined using a phase-contrast microscope to confirm the integrity of the chromosome spread. Then 45 µl drop of DAPI (4 µg/ml) diluted in McIlvaine's buffer and coverslip were applied to confirm the absence of remnant fluorescent signals under the epifluorescence microscope. At the same time, photographs of the chromosome spread were captured on each channel. This background picture was very useful to deduct the backgrounds from the pictures with fluorescent signals. The coverslip was removed by applying 70% ethanol, and dried. The slide was then ready for another round of *in situ* hybridization.

2.13 Physiological and agronomic evaluation

2.13.1 Septoria

Location and inoculum

The inoculum of *Septoria tritici* blotch was produced in the CIMMYT wheat pathology laboratory using a mixture of six aggressive strains with a spore suspension of 1 x 10^7 conidia/ml. The first inoculation of the germplasm was conducted between 28 and 30

days after planting i.e. four to five leaf stages. The inoculation was continued every week with in a total of three applications.

Field evaluation for seedling resistance

Field trials were conducted at CIMMYT's experimental stations in the State of Mexico, at Toluca (latitude 19°17′ N, longitude 99°40′ W, 2600m above sea level). All entries were grown with two replications at each location adjacent to each other in a complete completely randomized design. Each entry was sown in sets of 2 m rows with 4 g kernels per row.

Disease scoring for seedling resistance

Approximately four weeks after the last inoculation, disease severity was visually scored for each plot, using the double-digit scale (00–99) developed as a modification of Saari and Prescott's severity scale for assessing wheat foliar diseases (Saari and Prescott, 1975). The first digit (D1) indicates disease progress in canopy height from the ground level and the second digit (D2) refers to severity measured based on diseased leaf area. Both D1 and D2 were scored on a scale of 1 to 9. Disease evaluation was repeated three to four times at weekly intervals. For each evaluation, percentage disease severity was estimated based on the following formula:

% Severity = (D1/9) × (D2/9) × 100

The area under disease progress curve (AUDPC) was subsequently calculated using the formula:

$$\text{AUDPC} = \sum_{i-1}^{n}[\frac{(Y_i+Y_{(i+1)})}{2}\}x(t_{(i+1)} - t_i)]$$

Where Yi= STB severity at time ti, t(i+1) - ti = time interval (days) between two disease scores, n = number of times when STB was recorded (see Appendix 3).

2.13.2 Fusarium Head Blight

Location and inoculum

For inoculum production, a lima bean method similar to that of Buerstmayer et al. (2002) was used. First, the well-characterized *F. graminearum* isolates stored at -20ºC were transferred onto PDA medium and incubated for 7 days for reactivation. Five to seven pieces of medium were taken out with a sampler and put into a 250 ml flask

containing 100 ml of liquid lima bean medium and shaken for 6-7 days. The liquid medium was filtered with a sterile pledget and the flow through was placed at 4ºC for 12 hours. The sediment was collected and put into a 15 ml tube for centrifugation at 3000 rpm for 10 min. The pellet was suspended with 100 ml water in a 250 ml flask, from which 500 µl of suspension was taken and smeared on a plate of agar-beans medium (500 ml broth made from 20 g lima bean, 7.5 g agar), which was then incubated at 25ºC with a 12/12 hr day/night photoperiod. Seven days later, the fungal propagules were washed down from the plates into 2 L of distilled water and then the concentration was adjusted to 500,000 spores/ml using a haemocytometer. The stock suspension was stored at -20ºC until use, and then it was 10 times diluted to make a concentration of 50,000 spores/ml for field application.

Field evaluation for seedling resistance

Field trials were conducted at CIMMYT's experimental stations in the State of Mexico, at Toluca (latitude 19°17′ N, longitude 99°40′ W, 2600m above sea level). All entries were grown with two replications at each location adjacent to each other in a complete completely randomized design. Each entry was sown in sets of 2 m rows with 4 g kernels per row.

Disease scoring for seedling resistance

At anthesis, 10 spikes of each line (five per row) were tagged by colored sticky tape in the morning, and the lines were spray inoculated in the afternoon of the same day. For inoculation, precision CO2 backpack sprayers with flat fan nozzle were used, and the inoculum (50,000 spores/ml) was applied at a pressure of 40 psi and a rate of 39 ml/meter. The inoculation is repeated two days later.

At 25 dpi, FHB symptoms were scored on the 10-tagged spikes by counting the numbers of total and infected spikelets of each spike. The FHB index was calculated using the following formula: FHB index (%) = (Severity x Incidence)/100 (Stack and McMullen 1994), where Severity is the averaged percentage of symptomatic spikelets, and Incidence is the percentage of spikes which showed infection, e.g. a line with nine out ten spikes infected (90% incidence), and 20% of spikelets on average showing symptoms (20% severity), had an FHB index of 18% (90% * 20%) (see Appendix 4).

Chapter 3: Section I: Molecular cytogenetic characterization of novel wheat-*Th. bessarabicum* intercalary translocation lines

3.1 Introduction

Domestication, cultivation and thousands of years of selection have led to limited genetic variability in bread wheat, *Triticum aestivum* L. (2*n*=6*x*=42) compared to its wild relatives (Reif et al., 2005, Feuillet et al., 2008, Heslop-Harrison and Schwarzacher, 2012, Kishii et al., 2010, Borlaug, 1983, Van Hintun et al., 2000). However, Huang et al. (2007) suggested that modern plant breeding has resulted in changes of alleles present in the germplasm; but plant breeding has resulted in no apparent loss of allele numbers, or genetic diversity, in the investigated European wheat varieties over time.

There is enormous diversity in wheat landraces and within diploid, tetraploid and hexaploid wild relatives: Able and Langridge (2006) noted that as little 10–15% of the available gene pool has been exploited in cultivars. Simmonds (1993), Gale et al. (1989) and Schwarzacher et al. (1992) have discussed how alien introgression of chromosomes by hybridization with wild relatives can introduce desirable characters by crossing and backcrossing into cultivated species, and such chromosome engineering is important for crop breeders to broaden the genetic base of wheat (Friebe et al., 1996, Jiang et al., 1993, Sears, 1956, Lukaszewski, 1990, Wang, 2011). Introgression has two important steps: sexual hybridization commonly known as wide hybridization to bring the wild or 'alien' genome into a cultivated background followed by homologous and/or homoeologous recombination to facilitate pairing of normal wheat chromosomes with alien chromosomes to achieve successful transfer (Feuillet et al., 2008, Jauhar and Chibbar, 1999). Alien introgression dated back to 1930s when the first wheat-rye introgression lines were made (see Heslop-Harrison, 1990). The best example of alien introgression of chromatin from a relative into wheat was the 1BL.1RS chromosomal translocation, which became very common and popular in wheat breeding (Lukaszewski, 1990). Between 1991 and 1995, 45% of 505 commercial cultivars of bread wheat from 17 countries carried this wheat–rye translocation (Rabinovich, 1998).

The chromosome arm 1RS from rye carried genes conferring resistance to leaf rust *Lr26*, stem rust *Sr31* and powdery mildew *Pm8* (Kumar et al., 2003, Singh et al., 1990) and *Yr9* gene conferring resistance to stripe rust.

Alien introgression frequently carries deleterious alleles and/or genes, a phenomenon known as 'linkage drag' (Gill et al., 2011). The main reason is that recombination between an introgressed alien chromosome and its homoeologue is completely suppressed in the target gene region. In such cases standard recombination based approaches cannot be used, to overcome this 'recombination barrier' a special cytogenetic manipulation technique called 'chromosome engineering' (CE) is required (Feuillet et al., 2008, Gill et al., 2011). Alternative of alien introgression is transgenics, where gene required is identified and brought in. Another way is by producing somatic/cell fusion hybrids, which have not really used in cereals. More recently, CRISPR/gene editing added to the toolbox (Shan et al., 2014). However, wide hybrids have been the major impact on alien gene exploitation in cereals with worldwide use over the last half-century, including in the most successful and newly released modern wheat (e.g. Skyfall in Europe, first grown widely in 2015, and including *Aegilops ventricosa* genetics).

The construction of ILs harboring discrete, defined chromosome segments from the wild species, ideally representing a tiling path across the whole genome and within an otherwise uniform genetic background will improve our ability to perform accurate phenotyping, mapping, and ultimately cloning and combining minor and major QTLs for disease resistance from wild and alien species (Zamir, 2001, Wulf and Moscou, 2014).

3.1.1 Thinopyrum bessarabicum- Rich source of useful genes for wheat improvement

Species belonging to the *Thinopyrum* Dewey genus (also placed in genera including *Agropyron, Elytrigia, Lophopyrum*) have been described as potential sources of both biotic-stress disease resistance (Friebe et al., 1993, Knott, 1968) and abiotic-stress resistance (King et al., 1997). Since 1930 breeders have exploited different *Thinopyrum* species after NV Tsitsin and his colleagues initiated intensive hybridization programs and first showed that these species readily hybridized with various *Triticum* species (Chen, 2005, Tsitsin, 1960). Breeders have developed wheat-*Thinopyrum* hybrids to transfer these resistances into bread wheat (Graybosch et al., 2009, Friebe et al., 1992b,

Friebe et al., 1992a). *Th. bessarabicum* (2*n*=2*x*=14, JJ; elsewhere the designation E^bE^b is used) is a perennial, rhizomatous maritime wheatgrass distributed in the Black Sea and Mediterranean region. It possesses salinity tolerance and resistance to several diseases (Gorham et al., 1985, King et al., 1997, William and Mujeeb-Kazi, 1993). The development of wheat–*Th. bessarabicum* alien chromosome addition lines is providing germplasm for further utilization in wheat improvement (William and Mujeeb-Kazi, 1993) including the lines studied here. Several different salt tolerant wheat–*Th. bessarabicum* translocation lines T5AS·5JL where the translocation involved wheat chromosome arm 5AS and *Th. bessarabicum* chromosome arm 5JL were developed through homoeologous pairing induction in the absence of *Ph1* and identified using DNA markers (King et al., 1993). A translocation line T2JS-2BS·2BL involving chromosome 2J of *Th. bessarabicum* was developed and characterized by FISH (Qi et al., 2010).

Wheat-alien addition lines where a single pair of homoelogous chromosomes from a related or non-related species is added to the wheat complement, are used to identify alien chromosomes carrying useful genes and act as a starting point for the cytogenetic manipulation of alien genetic material to wheat. However, none of the alien chromosome addition lines has been used as a commercial variety because of the genetic instability of the alien chromosome and incorporation of undesirable characters linked with the alien chromosome (Hassani et al., 2010). Translocation lines involving small alien segments rather than complete chromosome are preferred and used directly by wheat breeders because they are genetically more stable carry less linkage drag, and have regular meiotic behavior (Falke et al., 2009). Developments of wheat–*Th. bessarabicum* translocations have been reported in earlier studies. Several different wheat–*Th. bessarabicum* translocation lines T5AS·5JL where the translocation involved wheat chromosome arm 5AS and *Th. bessarabicum* chromosome arm 5JL were developed through homoeologous pairing induction in the absence of *Ph1* and identified using DNA markers (King et al., 1993). Translocation line T2JS-2BS·2BL involving chromosome 2J of *Th. bessarabicum* was developed and characterized by fluorescence *in situ* hybridization (FISH) (Qi et al., 2010). Shen et al. (2013) reported the presence of the blue-grain gene (*BaThb*) in *Th. bessarabicum* on chromosome 4J.

This blue-grain trait then can be transferred to wheat and thus can be used for making specialty foods as well as food colorants. It also can be used as a visible marker in

genetics and breeding programs. Pu et al. (2015) used gamma-irradiation induce translocation lines to map this blue-grain gene (*BaThb*). This translocation lines allowed the deletion mapping of 101 4J-specific markers and fine mapping of blue-grained gene *BaThb.*

3.1.2 FISH and repetitive DNA sequences for chromosome identification

Fluorescent *in situ* hybridization of repetitive DNA sequences to chromosomes can result in chromosome specific banding patterns for studying chromosome behaviour, phylogenetic relationships and tracing chromosome rearrangements. The *dpTa1* clone is a repetitive DNA probe, which is D-genome specific (Rayburn and Gill, 1986, Bardsley et al., 1999) and it distinguishes the D-genome chromosomes from the A and B genome chromosome of hexaploid wheat. The DNA clone *pSc119.2* contains a 120 bp tandem repeated DNA sequence from rye (*Secale cereale* L.) (Bedbrook et al., 1980, Mcintyre et al., 1990) and can bind specifically with B genome chromosome of wheat. This repetitive probe has characteristic banding pattern, which could identify recombinant chromosomes. The GAA microsatellite gives unique hybridization patterns on many chromosome arms (Cuadrado et al., 2000, Cuadrado et al., 2008). Together, these probes can identify chromosome arms and translocations (Pedersen and Langridge 1997). The aim of present study was the detailed identification, cytological and molecular identification of chromosomes involved in translocations in a set of wheat-*Th. bessarabicum* translocation lines. Precise characterization of a wheat-alien recombinant chromosome is essential for effective utilization of novel traits in wheat breeding and subsequent tracking with DNA markers.

3.1.3 Aims

Here, the aim was the characterization of novel wheat-*Th. bessarabicum* intercalary translocation lines derived from an international breeding program using molecular cytogenetics or in situ hybridization with a range of repetitive and genomic DNA probes.

3.2 Materials and Methods

3.2.1 Plant material

Several *Thinopyrum bessarabicum* translocation lines produced in a Prinia (CIMMYT bread wheat) background in CIMMYT Mexico, and were initially selected for salt resistance. These lines were developed by manipulation of the *Ph* genetic control mechanism (Sears 1977). Seeds were obtained from Dr Masahiro Kishii (CIMMYT, Mexico) and the detailed pedigree of lines is mentioned in table 3.1.

All the lines included in this study are often referred to as a wheat-*Th. bessarabicum* hybrid. They were germinated in Petri dishes and grown in the growth cabinet facility under controlled conditions.

3.2.2 Chromosome preparation

Chromosome preparations were made from the root tips of plants using standard techniques. In brief, plant seeds were germinated on moist filter paper in Petri dishes for two days. After germination root tips were collected and incubated in ice-cold water for 24 h at 40 C before fixing in fresh 3:1; ethanol: acetic acid and left overnight. For preparation, roots were washed with the enzyme buffer for 5 min and then water for 1 min before digesting in an enzyme solution 0.1 % (w/v) cytohelicase (Sigma-Aldrich, Steinheim, Germany), 0.1 % (w/v) cellulase Onozuka RS (Sigma- Aldrich), 0.1 % Pectolyase Y23 (Sigma-Aldrich) in 10 mM citrate buffer, pH 4.8 for 90 min at 37 ºC. After digestion the enzyme solution was replaced with the enzyme buffer, and then a single root tip was squashed in 60 % (v/v) acetic acid under a stereomicroscope on a clean glass slide (SuperFrost®, Menzel- Glaser, Thermo Scientific). The root cap and other permanent tissues were removed by using fine needles and forceps. The squash was covered with a 18mm x 18mm coverslip and thumb pressure was applied. The slide was briefly passed over the flame to help disperse the cytoplasm. After freezing, coverslip removal and dehydration through an alcohol series, slides were selected under phase contrast or after staining with 4', 6'-diamidino-2-phenylindole (DAPI, Sigma), dehydrated, dried, and stored at -20 °C until hybridization.

3.2.3 Fluorescent in situ hybridization

DNA *in situ* hybridization followed the method described by Schwarzacher and Heslop-Harrison (Schwarzacher and Heslop-Harrison, 2000b) with minor modification. The probe mixture contained 50% (v/v) formamide, 20% (w/v) dextran sulphate, 2 x SSC, 25-100 ng probe, 20 μg of salmon sperm DNA and 0.3% SDS (sodium dodecyl sulfate) as well as 0.12 mM EDTA (ethylene-diamine-tetraacetic acid) and autoclaved total genomic DNA from wheat "Chinese Spring" as blocking DNA at 4-20 x probe concentration. Probe and chromosomal DNA was denatured together on a Hybaid Omniblock for 7 minutes at 78°C and slowly cooled to the hybridization temperature of 37°C. Washes were carried out with 0.1 x SSC at 42°C at an equivalent to 80% stringency. Hybridization sites were detected with 2.0 μg/ml streptavidin conjugated to Alexa594 (Molecular Probes) and 4 μg/ml antidigoxigenin conjugated to FITC (fluorescein isothiocyanate) (Roche Diagnostics). Chromosomes were counterstained with 0.2 μg/ml DAPI diluted in McIlvaine's buffer pH7 and mounted in antifade solution (Citifluor). Preparations were analysed on a Zeiss epifluorescence microscope single band pass filters equipped with a CCD camera (Optronics, model S97790) and overlaid using Adobe Photoshop CC. Only those functions that treat all pixels of the image were used. For clarity in fig. 3.6, 3.14, 3.20, 3.25, 3.29, 3.36 some probe colours were reversed so the genomic *Th. bessarabicum* probe is always shown in red. Between 10 and 20 metaphases were analyzed for each line. To obtain measures of total karyotypic lengths (in pixels), karyotypes from different metaphases were prepared and measured in Nikon NIS 3.2 software.

Table 3.1: Wheat, *Th. bessarabicum* and translocation lines in a Prinia[1] wheat background used in the study

Line	Source	Description/Cross	Chromosome Number (2*n*)
CS	Sears, Missouri	Chinese Spring (bread wheat)	42
Th. bessarabicum	Genebank, (USDA-ARS) PI 531711 (France)	*Thinopyrum bessarabicum*	14
CSPh1	CIMMYT	Chinese Spring *ph1b* mutant line	42
1160	CIMMYT	CS/TH.BESS//CSph/3/4*PRINIA[2]	42
1164	CIMMYT	CS/TH.BESS//CSph/3/4*PRINIA	42
1168	CIMMYT	CS/TH.BESS//CSph/3/4*PRINIA	42
1172	CIMMYT	CS/TH.BESS//CSph/3/3*PRINIA	42
1176	CIMMYT	CS/TH.BESS//CSph/3/2*PRINIA	42
1180	CIMMYT	CS/TH.BESS//CSph/3/3*PRINIA	42
Ph - pairing homeologous gene; Prinia- CIMMYT bread wheat			

1) The pedigree of Prinia is PARULA/VEERY-6//MYNA/VULTURE; 'Veery' carries the 1RS.1BL translocation (from varieties Bezostaya through Kavkaz); Prinia also carries the rye translocation, which is a widespread translocation in CIMMYT wheats with 'Veery' in their pedigree.

2) In full form, the pedigree of 1160 is [(CS x *Th. bessarabicum*) x CSph] x Prinia backcrossed three more times to Prinia; subsequent generations have been selfed and bulked.

3.2.4 Probes used

For *in situ* hybridization, total genomic DNA was isolated from young leaves using CTAB method (Doyle and Doyle, 1990).

FISH was carried out using the following repetitive sequences:

- ***pTa535*:** contains a 342 bp tandemly repeated DNA isolated from Chinese Spring (*Triticum aestivum*) (Komuro et al., 2013).
- ***pSc119.2*** contain a 120 bp tandemly repeated DNA sequence isolated from *Secale cereale* (McIntyre et al., 1990).
- ***pTa71*** contains a 9 kb *Eco*RI fragment of the repeat unit of 25S-5.8S-18S rDNA isolated from *T. aestivum* (Bedbrook et al., 1980).
- ***dpTa1*** containing tandem repeat with a monomeric length of 340bp isolated from *T. aestivum* was subcloned by Vershinin et al. (1994) and is homologous to *pAs1*(Rayburn and Gill, 1986), *pHcKB6* from *Hordeum chilense* (Anamthawat-Jónsson and Heslop-Harrison, 1993) and the *Afa* family (Nagaki et al., 1995, Nagaki et al., 1998).
- ***pTa794*** contains a 410 bp fragment of 5S rDNA of *T. aestivum* (Gerlach and Dyer, 1980).
- **GAA microsatellite** GAA satellite sequences were amplified from the genomic DNA of *Hordeum vulgare* and labelled with biotin-16-dUTP using PCR (Vrána et al., 2000).
- **Genomic DNA probes** Genomic DNA from the species *Th. bessarabicum* and *T. monococcum* was first sheared in an autoclave at 110º for 3 min and was labelled by random priming.

3.3 Results

3.3.1 Characterization of wheat-Th. bessarabicum recombinant lines

The wheat-*Thinopyrum bessarabicum* translocation lines with the pedigrees shown in table 2.1 were stable and included characteristics of the *Th. bessarabicum* ancestor. In metaphase chromosome preparations, the labelled genomic DNA of *Th. bessarabicum* revealed the alien chromosome segments in the lines, and the repetitive DNA probes allowed identification of wheat and recombinant chromosomes. The hybridization

patterns of the repetitive DNA probes were largely consistent with published karyotypes (Danilova et al., 2012, Kubaláková et al., 2005), allowing identification of most of the chromosomes. All lines had 2*n*=42 with 21 pairs of homologous chromosomes and alien derivatives were always disomic. There was no evidence for inter- or intra-genomic recombination in any of the lines despite the *ph1b* background.

To characterize the *Th. bessarabicum* recombinant chromosomes, simultaneous GISH and FISH was performed using genomic and repetitive DNA probes on spread metaphase chromosome fixed onto a glass slide. The unique banding patterns of repetitive DNA probes were helpful in identifying and characterizing the recombinant chromosome.

Line 1160: Recombinant chromosome T4BS.4BL-4JL

A small, terminal segment labelled with *Th. bessarabicum* genomic DNA represented 10% of the long arm of a wheat chromosome and 1.2% of the total karyotype length (Fig. 3.6). There was no *dpTa1* signal on the translocated chromosome (Fig. 3.1, 3.2), and further FISH experiment using the DNA clone *pSc119.2* showed two strong intercalary sites (Fig. 3.3, 3.4), proximal to the alien chromatin on the long arm, and one distal site on the short arm of the wheat chromosome. The GAA microsatellite showed several strong signals at the centromere and two minor bands on the long arm of the recombinant wheat chromosome (Fig. 3.5). The staining pattern of wheat chromosomes as shown by GAA microsatellite was similar to staining pattern described in Danilova et al. (2012) and was used as a reference. The GAA pattern confirms that chromosome involved in translocation is 4B. The probe patterns determine the translocation T4BS.4BL-4JL (Fig. 3.6). This recombinant wheat chromosome was identified as 4B (Fig. 3.6) and the translocation designated as a T4JS-4BS.4B.

Line 1164: Recombinant chromosome T6BS.6BL-6JL

The identification of lines 1164 was easy and straightforward. FISH with *Th. bessarabicum* identified a terminal *Th. bessarabicum* segment represented 35% of the long arm (3.6% of the total karyotype length) of the recombinant chromosome (Fig. 3.14). The recombinant wheat chromosome carried a major 45S rDNA (*pTa71*) site on the short arm that was also visible as a constriction with DAPI (3.10, 3.11), and *pSc119.2* revealed one strong intercalary and one strong terminal site on the long arm

(Fig. 3.7). After observing characteristic DAPI bands, we thought it carries a 1BL.1RS chromosome, to confirm this same slide was re-probed using rye genomic DNA, which clearly labelled and confirmed the presence of 1BL.1RS translocation (Fig. 3.8, 3.9). Use of GAA further helps to identify and confirm translocated chromosome 6B, multiple GAA bands were present around the centromere (Fig. 3.12, 3.13). The translocated chromosome was thus identified as T6BS.6BL-6JL (Fig. 3.14).

Line 1168: Recombinant chromosome T5AS.5AL-5JL

The *Th. bessarabicum* chromosome segment was revealed to represent 25% of a long wheat chromosome arm (2.8% of the total karyotype length; Fig. 3.20). The genomic DNA probe labelled the introgressed segment strongly, and also the D-genome chromosomes throughout their lengths with stronger hybridization to repetitive sequences as sites corresponding to the repetitive satellite *dpTa1* (Fig. 3.15). Neither *dpTa1* nor *pTa71* signal was detected on this recombinant wheat chromosome; this confirms that chromosome involved in translocation is not the D and B-genome chromosome (Fig. 3.15, 3.16).

Multicolour GISH was performed using both *Th. bessarabicum* and *T. monococcum* genomic DNA as a probe, which could identify all fourteen labelled A-genome chromosome arms along with alien chromatin (Fig. 3.17). To determine which A-genome chromosome was involved in the translocation FISH using *pSc119.2* as a probe was done. One chromosome showed strong terminal *pSc119.2* single on the short arm and alien chromatin on the long arm (Fig. 3.18, 3.19). This signaling pattern of *pSc119.2* was similar to the signaling pattern described in Kubaláková et al. (2005), which confirms that the recombinant chromosome was 5A (Fig. 3.20).

GAA microsatellite hybridize to some A genome chromosome of wheat could be useful identify A genome chromosome (Danilova et al., 2012). There are some small GAA sites on the A chromosomes, with the exception of 1A, and on chromosomes 1D, 2D and 7D (Kubaláková et al., 2005). We use GAA microsatellite as a FISH probe which showed a minor band at centromere and *Th. bessarabicum* chromatin on the long arm (Fig. 3.20), which confirmed that chromosome involved in translocation was 5A (Fig. 3.20).

Line 1172: Recombinant chromosome T5DL.5DS-5JS

FISH analysis revealed the small *Th. bessarabicum* fragment at the distal end of the small arm of a medium sized wheat chromosome (35% of the arm and 2.5% of the total karyotype length). A minor 45S rDNA site was visible at the border between the alien chromatin and wheat chromosome (Fig. 3.21, 3.22). Two large *pTa71* sites are visible at chromosomes 1B and 6B and so the minor 45S rDNA site most likely identifies chromosome 5D (Fig. 3.22).

To confirm, dual colour FISH was done using repetitive DNA clone *dpTa1*, and *Th. bessarabicum* showed five distinctive bands on the long arm and two on the short arm (Fig. 3.23, 3.24), which clearly identify it as a 5D (Fig. 3.25).

Line 1176: Recombinant chromosome T2BS.2BL-2JL

A small *Thinopyrum* segment (45% of the arm and 2% of the total karyotype length) was revealed on the short arm of one wheat chromosome pair. In many cases, a distinctive gap is also visible along the *Th. bessarabicum* labeling (Fig. 3.26). The long arm of the wheat chromosome had minor *pSc119.2* sites (Fig. 3.26, 3.27) and a strong GAA signal at the centromere and two minor signals on the short arm (Fig. 3.28). The translocated chromosome was identified as T2BS.2BL-2JL (Fig. 3.29).

Line 1180: Recombinant chromosome T1JS.1AL

The *Th. bessarabicum* probe revealed the presence of a whole arm of *Th. bessarabicum* chromosome (representing 2.5% of the total karyotype length) fused with a complete wheat chromosome arm, together forming a Robertsonian translocation. Both the wheat and *Thinopyrum* chromosome ends of the rearranged chromosome carried a minor *pSc119.2* site (Fig. 3.34, 3.35). Notably there is a heteromorphism with only one of the two long (wheat-origin) arms of the translocated chromosome carrying a minor *pSc119.2* site. The 45S rDNA probe (*pTa71*) showed the expected major sites on chromosomes 1B and 6B, and minor sites on 5D (Fig. 3.30, 3.31). A single minor site was visible on the *Thinopyrum* labelled chromosome arm. A genomic probe from *T. monococcum* weakly labelled the wheat arm involved in the rearrangement (Fig. 3.32, 3.33) revealing that the translocated wheat arm belongs to the A-genome of wheat. This finding together with the fact that the minor *pTa71* site, characteristic to the 1AS, was missing from any of the remaining A-genome chromosomes suggested that the whole

short arm of wheat chromosome 1A had been replaced by the *Th. bessarabicum* arm, so the translocation was assigned as T1JS.1AL (Fig. 3.36).

3.3.2 Repetitive sequence locations in Prinia wheat

As a reference, *in situ* hybridization with GAA and *pSc119.2* was carried out to Prinia chromosomes (Fig. 3). Probes showed characteristic hybridization patterns on all B genome and some A and D genome chromosomes, and could be identified by comparison with Kubaláková et al. (2005) in a tetraploid wheat and Danilova et al. (2012) in the Chinese Spring and Canthach hexaploid wheats, showing minor variations. The DAPI staining of terminal heterochromatin (shown in red in Fig. 3.37) and *pSc119.2* hybridization pattern identified the 1RS chromosome arm in Prinia.

3.3.3 Agronomic performance

Evaluation of Septoria tritici resistance

All the lines were almost susceptible to *Septoria tritici* (Appendix 3). Parent Prinia showed some resistance. Line 1176 was most susceptible than others. No previous data have been reported stating Septoria resistance in *Th. bessarabicum*. No lines had promising resistance to Septoria.

Evaluation of Fusarium Head Blight (FHB) resistance

Translocation line 1164 was very resistant to FHB, and so does 3J disomic addition line (2*n*=44); this could be novel resistance. Parent Prinia showed limited resistance to FHB (Appendix 4).

3.4 Discussion

We have identified and characterized six novel wheat-*Th. bessarabicum* translocation lines originating from a program involving backcrosses into wheat of 'Chinese Spring' wheat x *Th. bessarabicum* amphiploid and the Chinese Spring *Ph1* mutant. The progenies were backcrossed up to four times to the CIMMYT wheat 'Prinia' (Table 1). Notably, five of the six translocations involved distal alien chromosome segments representing less than half the chromosome arm, and between 1.2 and 3.5% of the whole genome (Fig. 3.6, 3.14, 3.20, 3.25, 3.29, 3.36).

These terminal translocations involved four different homoeologous groups on all three genomes (4B, 6B, 2B, 5A and 5D). The sixth line analyzed here was a whole arm T1AL.1JS translocation (Fig. 3.36).

The repetitive DNA probes enabled identification of the wheat chromosomes involved in the translocations. To assist with chromosome identification and show relationships of the genomes, no blocking DNA was used in some of the experiments with *Thinopyrum* genomic DNA probe. The D-genome chromosomes all hybridized weakly throughout their length, showing the close relationship of the dispersed sequences between the J and D genomes. It was also notable that the regions of the D-genome chromosomes homologous to the *dpTa1*/*pHcKB6/Afa* sites were labelled with the J genomic DNA probe when no blocking was used (Fig. 3.15, 3.16), indicating that this sequence family is the primary, highly abundant tandemly-repeated DNA family in the *Thinopyrum* genome. When wheat-blocking DNA was used (Fig. 3.15, 3.16) hybridization to the D-genome chromosomes was substantially reduced, and the *dpTa1* sites were not labelled (hybridization sites would have also been competed for by the *pTa71* probe used in this slide).

Alien chromosome introgression can involve substitution of whole chromosomes. Lines are obtained relatively easily by backcrossing an amphiploid hybrid derivative to the wheat parent, and such lines are available as cytogenetic stocks for a number of alien species; addition lines may also be selected from these crosses (Molnár-Láng et al., 2000, 2012). As noted in the introduction, linkage drag means such lines are not normally grown as varieties (Falke et al., 2009, Feuillet et al., 2008, Gill et al., 2011), and further crosses are needed to reduce the size of the alien chromosome fragment. Spontaneous whole-arm translocations can occur, such as the 1RS.1BL translocation found in many biscuit or feed wheat varieties (Heslop-Harrison et al., 1990) and occasional recombinants with small alien chromosome segments have been reported e.g. the Danish wheat variety ‘Viking’ (Schlegel et al., 1993) or the Portuguese wheat landrace ‘Barbela’ (Ribeiro-Carvalho et al., 1997) including a small terminal rye chromosome segment on chromosome 4B and 2D respectively. These occurrences are rare and unpredictable, and more directed approaches are needed. There has been interest in generating small alien translocations in introgression lines since the 1950s, first using ionizing radiation (Sears, 1956) and then the homoeologous pairing *Ph* mutation, which allows intergenomic pairing of homoeologous chromosomes, as used

here, or the 5BL deletion lines (Riley, 1958, Riley and Chapman, 1967b, Riley and Chapman, 1967a). However, because of background translocations and deletions (Comai, 2000), relatively few of these lines have been exploitable in breeding programs.

In some cases, the alien segment may initially be detected only by plant morphology and subsequently proven by molecular cytogenetic methods (e.g. Barbela), while no alien segment may be seen with *in situ* hybridization but only DNA markers indicate its presence (e.g. transfer to wheat of *Lr57* and *Yr40* from *Ae. geniculata,* Kuraparthy et al. (2007)). Background wheat translocations and deletions of chromosome segments, as well as aneuploidy, are also found in wheat hybrid genetic stocks. These may be falsely considered as candidates for including alien translocations because of their exceptional morphology and because they are missing wheat DNA markers or be present in addition to the alien segment causing instability. Intercalary recombinant chromosomes will normally carry the wheat centromere sequences, and hence may be more stable than alien addition chromosomes. Here we noted a constriction in the alien segment in line 1176 (Fig. 3.26, 3.27), and it may be that this can act as a neocentromere like the regions described by Kishii et al. (2001) in *Leymus racemosus*-wheat addition lines and Carvalho et al. (2008) in Triticale x Tritordeum hybrids. . Early generations of wide hybrid-derivatives carrying desirable traits may not reveal deleterious characters (Castilho et al., 1996), but performance penalties will become obvious to breeders in trials (Sepsi et al., 2008). Small terminal chromosome segments in wheat may carry a disproportionately high number of the genes on a chromosome (Heslop-Harrison, 1991).

Introgression of chromosome segments from the genus *Thinopyrum* has been of considerable recent interest, with reports from hexaploid (Graybosch et al., 2009, Liu et al., 2013) and diploid (2x) species such as *Th. elongatum* (Fu et al., 2012, Hu et al., 2012) but work with *Th. bessarabicum* (2x) has been more limited (King et al., 1997, William and Mujeeb-Kazi, 1993). Here, each characterized translocation line carries a *Th. bessarabicum* chromosome segment transferred to its homoeologous wheat chromosome (Fig. 3.1-3.36). Studies have revealed about 18 novel disease resistance genes introgressed from *Thinopyrum* species to bread wheat using both irradiation treatment and homoeologous recombination (Fedak and Han, 2005). Irradiation treatment was used by (Knott, 1961) and Sharma and Knott (1966) to transfer the stripe

resistance gene *Sr26* from the long arm of group 6 chromosomes of *Th. elongatum* to the long arm of wheat chromosome 6A. Sears (1973, 1977) transferred the *Lr19* leaf rust resistance gene from *Th. elongatum* to wheat by homoeologous recombination. A wheat streak mosaic virus resistance gene *Wsm1* was transferred to wheat from *Th. intermedium* (Graybosch et al., 2009, Liang et al., 1979, Mutti et al., 2011,) and has been characterized cytogenetically (Ali, 2012). The genomic constitution of the leaf rust resistant wheat-*Th. ponticum* partial amphiploid BE-1 has been characterized using FISH and GISH (Sepsi et al., 2008). An advanced approach by Ayala-Navarrete et al. (2007, 2013) involved pyramiding two desirable resistance genes (*Sr25* and *Lr19*) from *Th. intermedium* and *Th. ponticum* to combine both alien genes on the distal positions on chromosome arm 7DL, giving a trigenomic recombinant chromosome (pontin lines). The *Thinopyrum* genus also has genes of interest for abiotic stress resistance including salinity tolerance (Wang et al., 2003a, b), the target character in the lines studied here. The lines will also carry novel biotic resistances: for example, the T4BS.4BL-4JL line (Fig. 3.3, 3.6) has a similar translocation position to the wheat-rye translocation line T4BS.4BL-4RL carrying powdery mildew resistance (An et al., 2013).

Alien introgression lines are usually selected in the field based on their differences from each other (chromosome additions and substitutions) and for the traits (in particular biotic or abiotic stress resistance) that are desirable in breeding lines. Here, early generations were selected for high fertility plants with 42 chromosomes rather than any particular trait. This will select against background and intra- or inter-genomic translocations within and between the wheat A, B and D genomes, which were not seen in the lines since all non-translocation chromosomes showed *in situ* hybridization patterns expected. However, the strong selection for fertility might have been expected to favor particular chromosome-segment substitutions, so it is interesting that the five lines obtained here involve four homoeologous groups.

Yield and drought trials of the lines are now underway, but as yet no resistance has been noted to the fungal diseases FHB (Fusarium Head Blight) or Septoria (Kishii, unpublished). Where there is strong selection for introgression of traits located on particular known chromosome arms, substitution lines can be used in the parentage: for example, Castilho et al. (1997) used lines based on a 1U(1B) substitution line that was crossed to the *ph1b* mutant to introgress chromosome segments carrying high molecular weight glutenin genes into wheat (Brown et al., 1979, Islam-Faridi, 1988).

Despite several crossing strategies and six independent crosses giving the required transfer, only two types of intercalary wheat-*Ae. umbellulata* recombination events were detected in these lines. However, cytogenetic work is laborious and time-consuming. Furthermore, structural changes are difficult to recover in an enlarged population if they cannot be found in an earlier generation because of the potential problems incurred in chromosome preparation, chromosome banding and GISH/FISH. It is necessary to develop a credible marker method for efficient detection of structural aberrations.

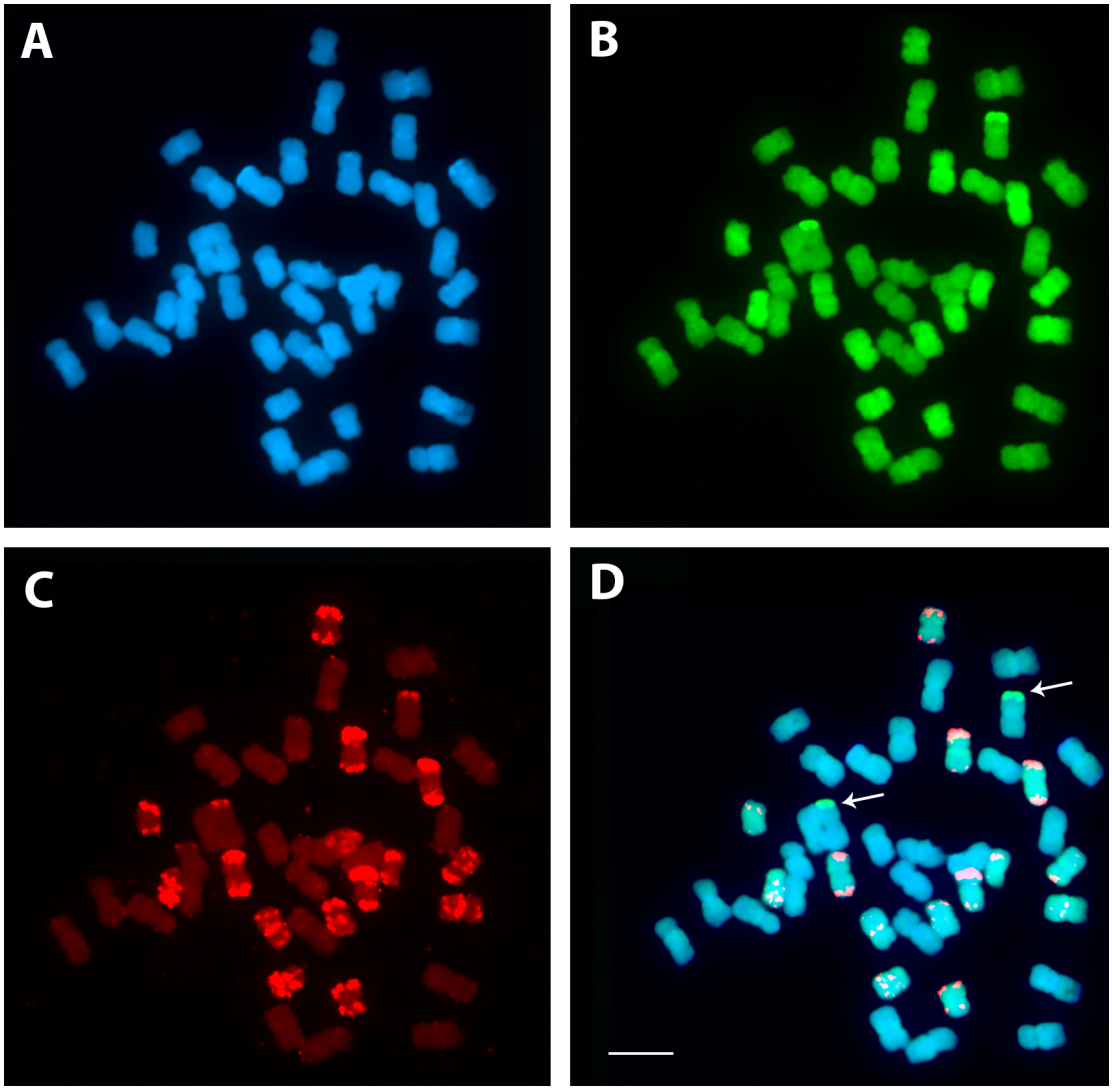


Fig. 3.1: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1160 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization allows detection of *Th. bessarabicum*-origin chromosome segment. (C) Hybridization pattern of the *dpTa1* clone labeled with biotin-16-dUTP (detected in red) which preferentially label D genome of wheat. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

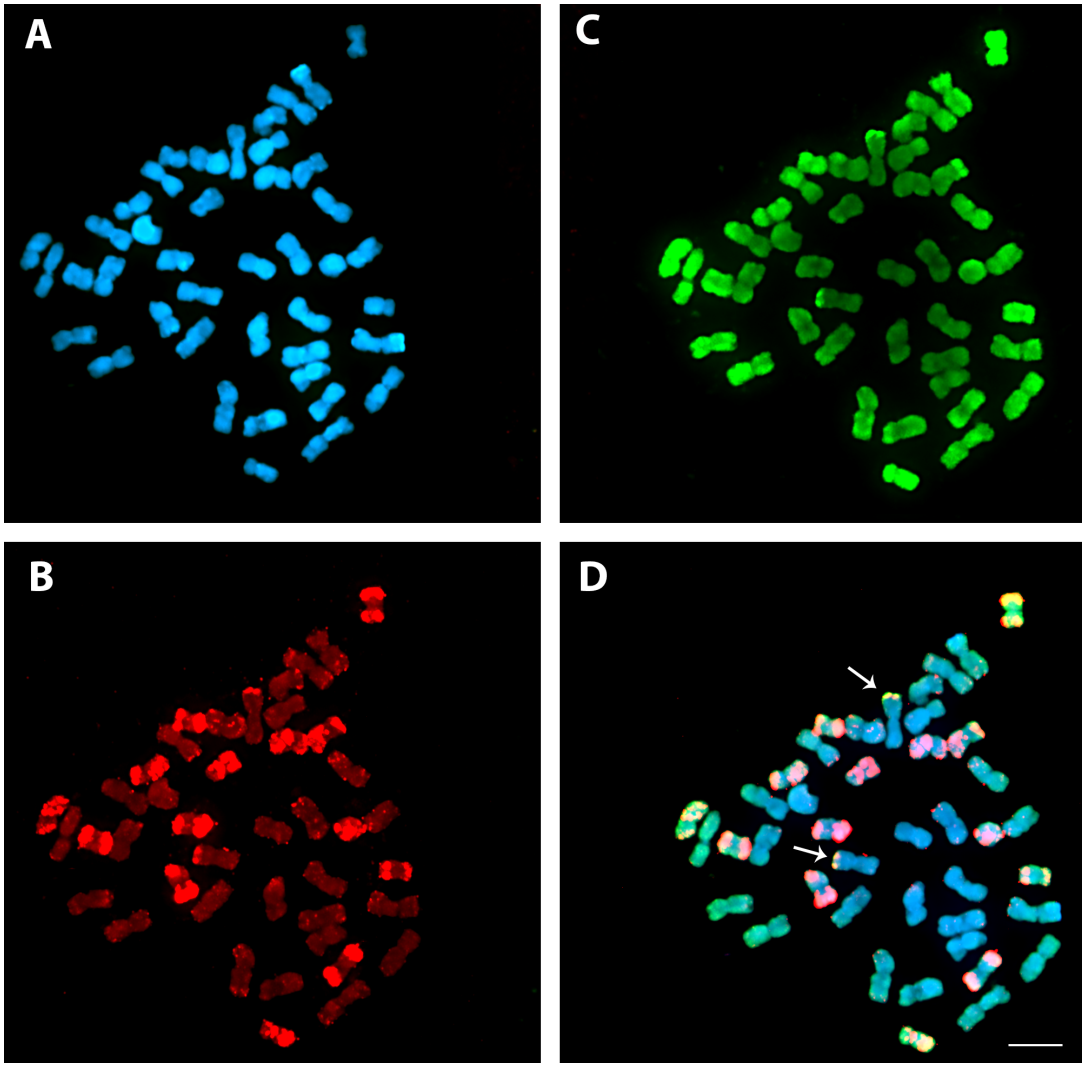


Fig. 3.2: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1160 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization allows detection of *Th. bessarabicum*-origin chromosome segment. (C) Hybridization pattern of the *dpTa1* clone labeled with biotin-16-dUTP (detected in red) which preferentially label D genome of wheat. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

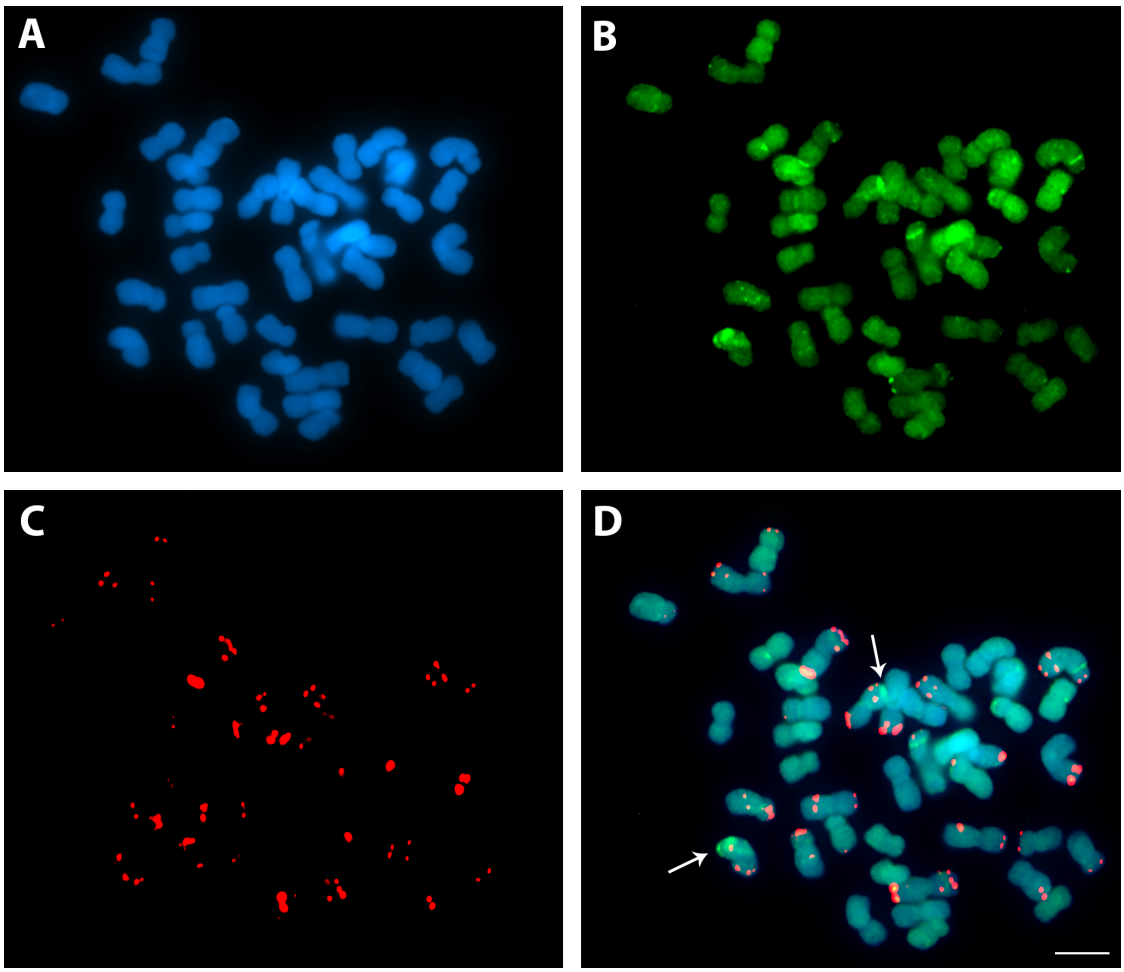


Fig. 3.4: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1160 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green), which allows detection of *Th. bessarabicum* origin chromosome segment. (C) Hybridization pattern of the *pSc119.2* DNA sequence labeled with biotin-16-dUTP (detected in red) that hybridize preferentially to B and some A-genome chromosomes. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

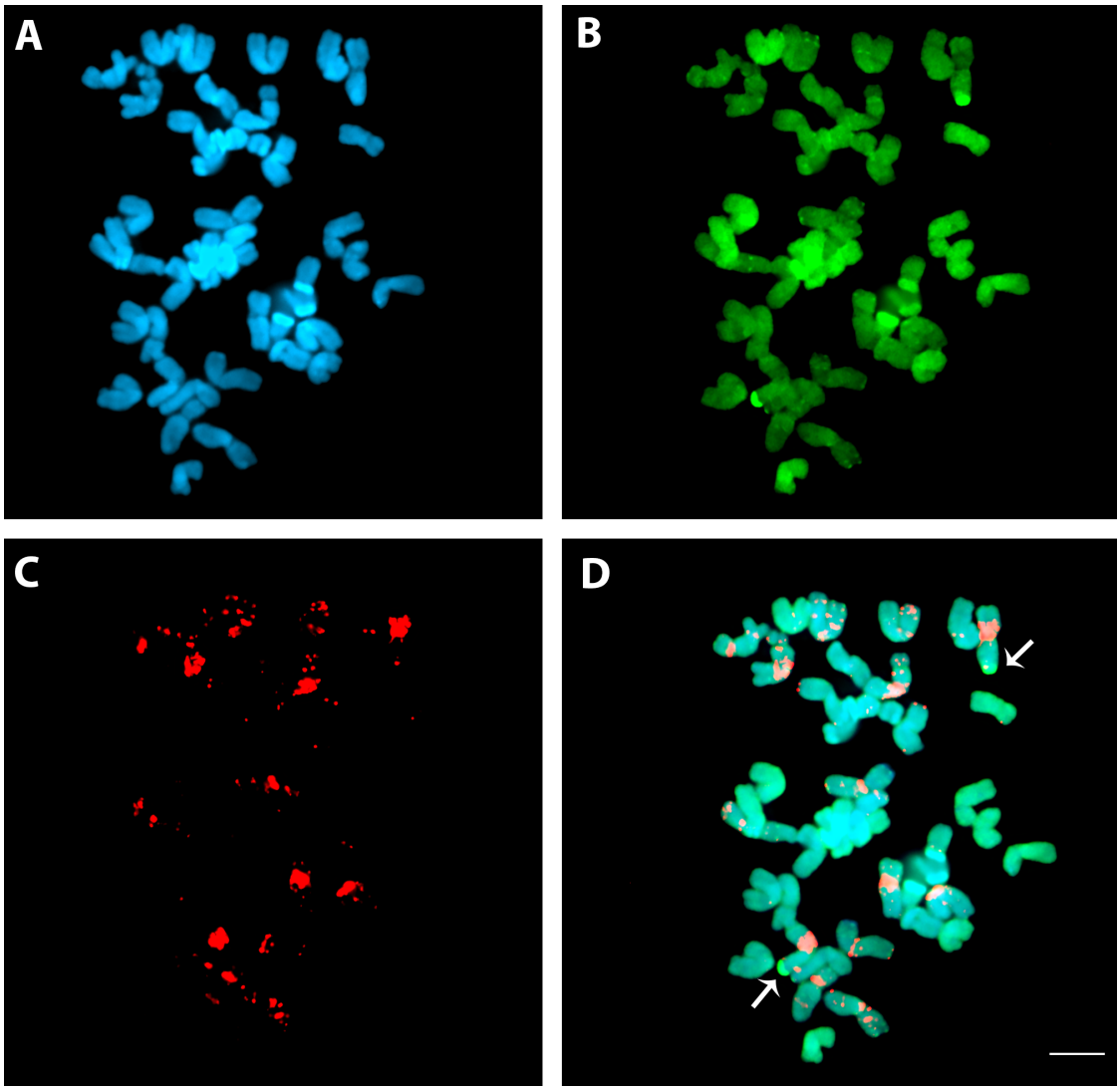


Fig. 3.5: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1160 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green), which allows detection of *Th. bessarabicum* origin chromatin segment. (C) Hybridization pattern of the GAA microsatellite sequence biotin-16-dUTP (detected in red) that hybridize preferentially to B-genome and some A-genome chromosomes. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

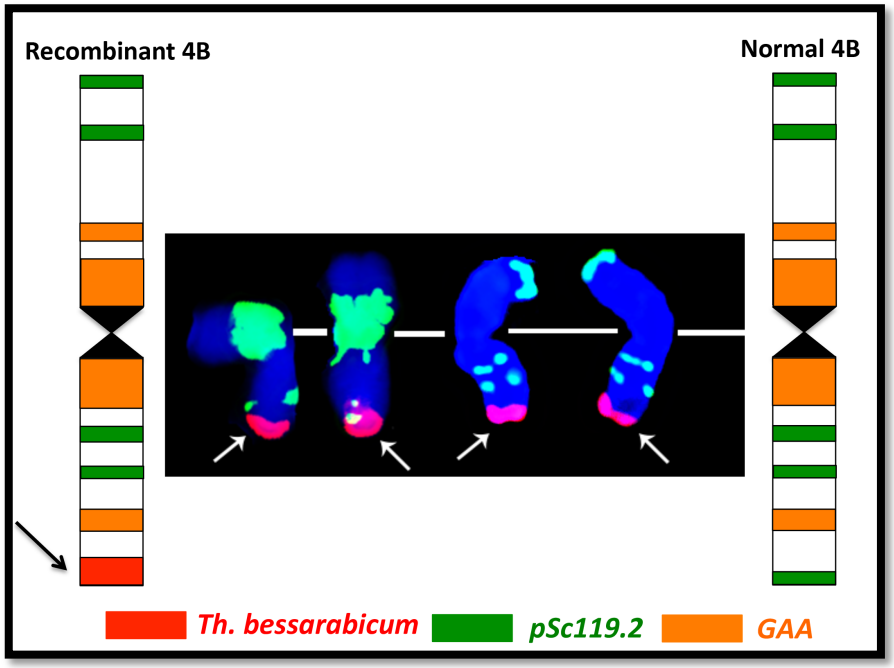


Fig. 3.6: Identification and schematic representation of normal and recombinant wheat chromosome 4B. Sketch of the normal wheat chromosome 4B (right) shows the unique pattern of *pSc119.2* (green), GAA (yellow). The recombinant wheat chromosome 4B (left) shows identical banding pattern of *pSc119.2* and GAA, except the distal band of *pSc119.2* on the long arm is lost due to the translocation of *Th. bessarabicum* chromatin (red).

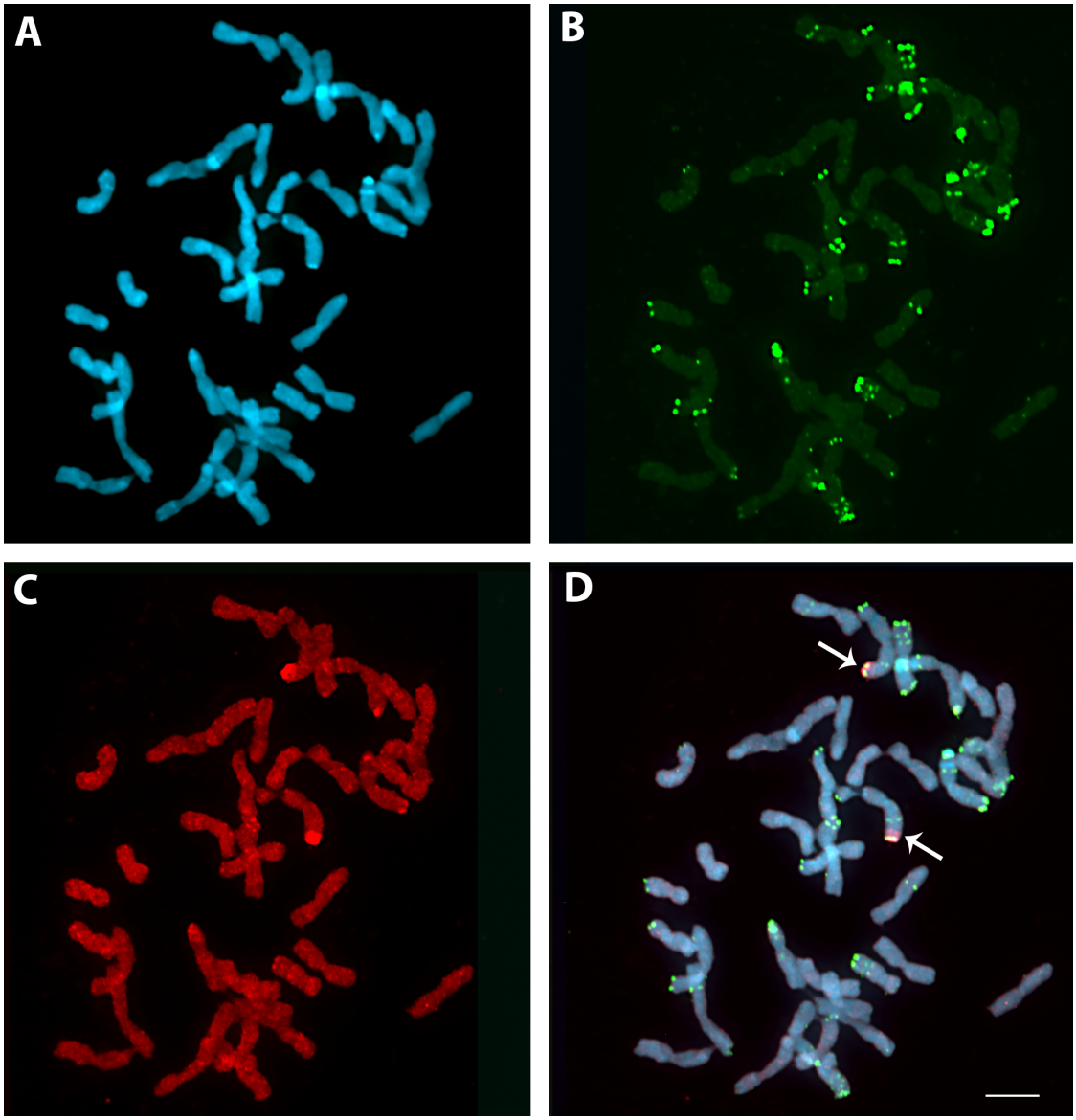


Fig. 3.7: Root tip metaphase chromosome of the wheat-*Th. bessarabicum* introgression line 1164 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) Hybridization pattern of the *pSc119.2* DNA sequence labeled with digoxigenin-11-dUTP (detected in green) that hybridize preferentially to B-genome and some A-genome chromosomes. (C) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with biotin-16-dUTP (detected in red), which allows detection of *Th. bessarabicum* origin chromosome segment. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

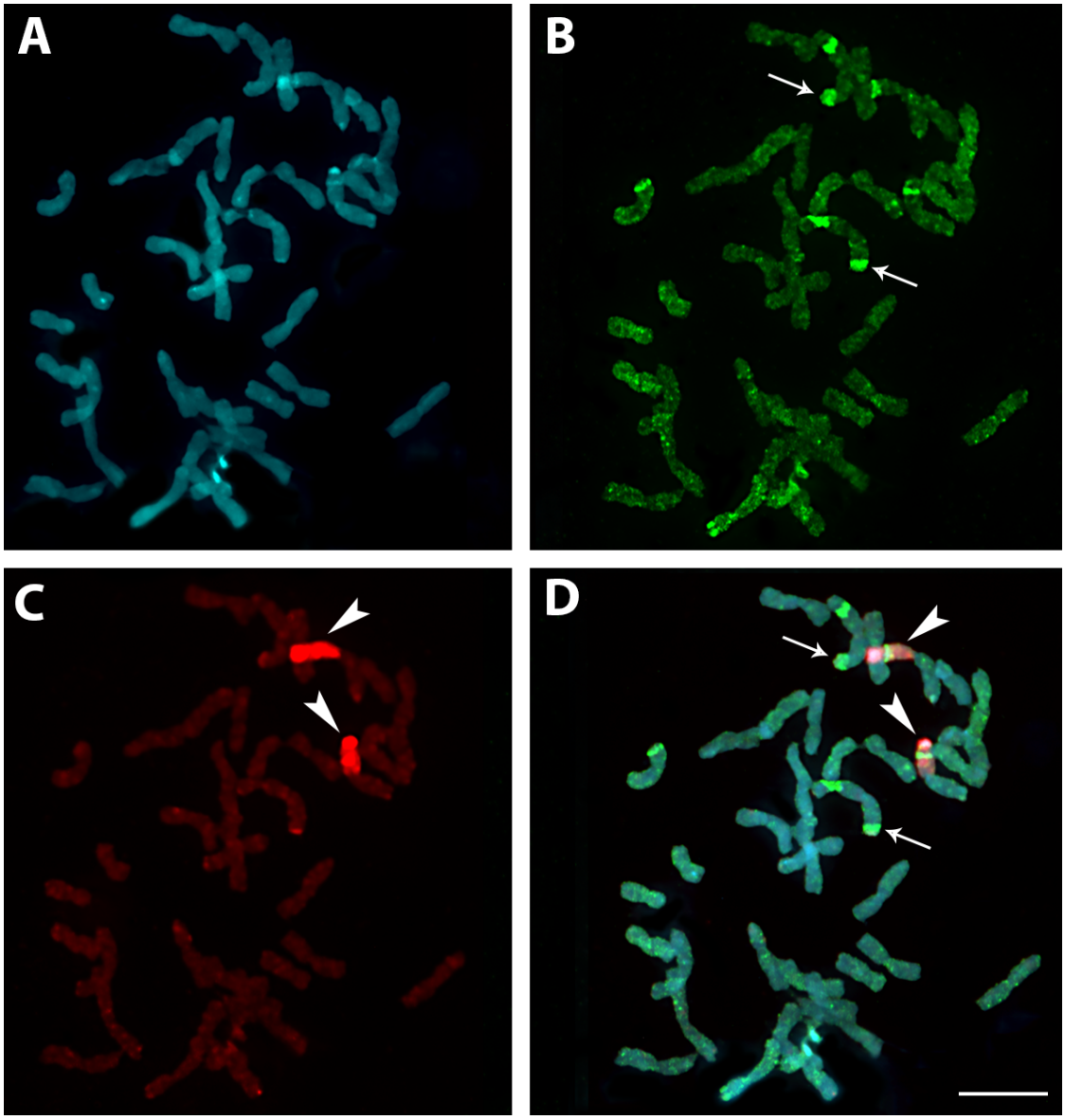


Fig. 3.8: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1164 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green), which allows detection of *Th. bessarabicum* origin chromosome segment. (C) *In situ* hybridization of the total genomic DNA from *Secale cereale* with biotin-16-dUTP (detected in red), which allows identification Rye origin chromosome segment (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

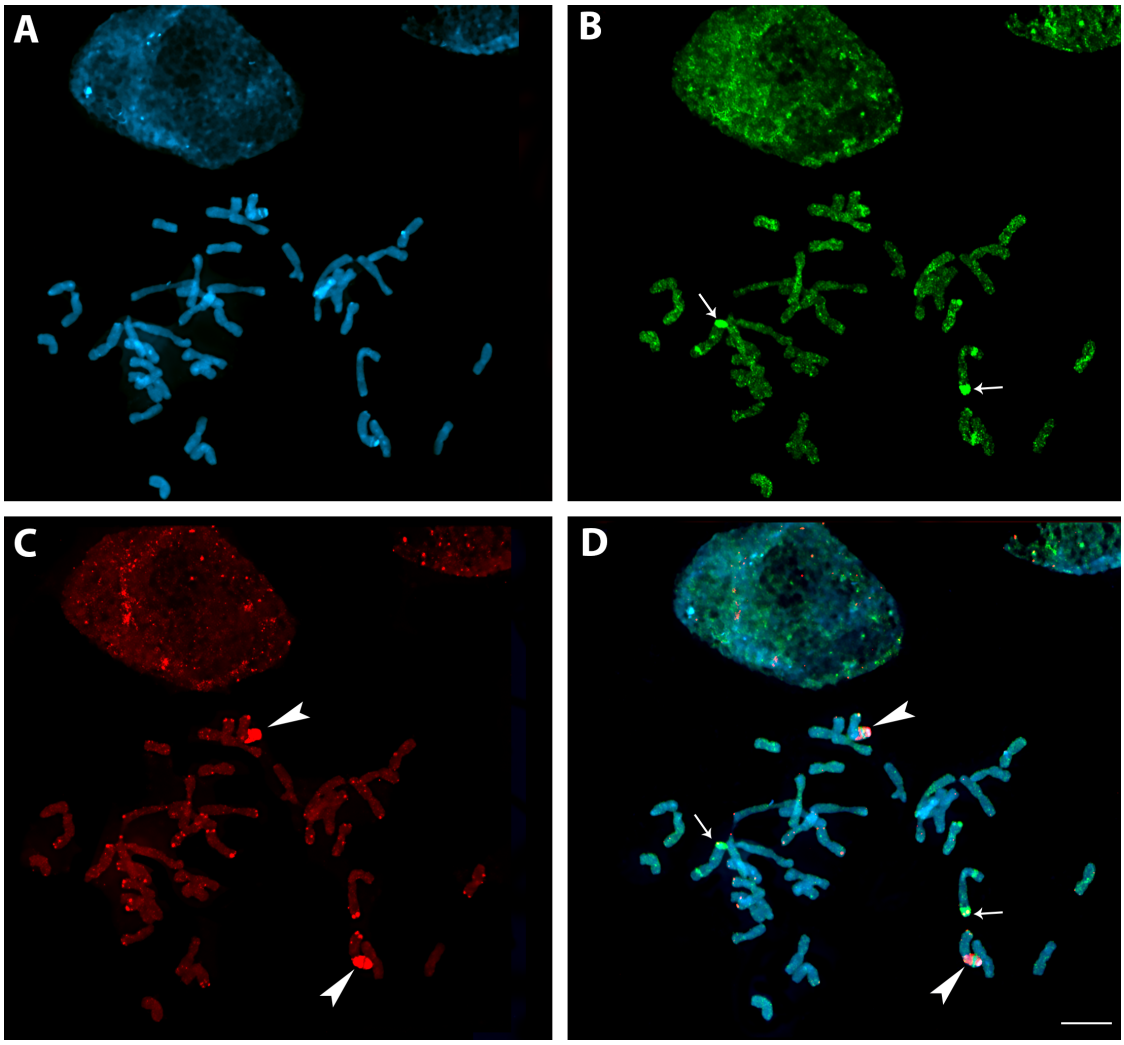


Fig. 3.9: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1164 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green), which allows detection of *Th. bessarabicum* origin chromosome segment. (C) *In situ* hybridization of the total genomic DNA from *Secale cereale* with biotin-16-dUTP (detected in red), which allows identification Rye origin chromosome segment (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

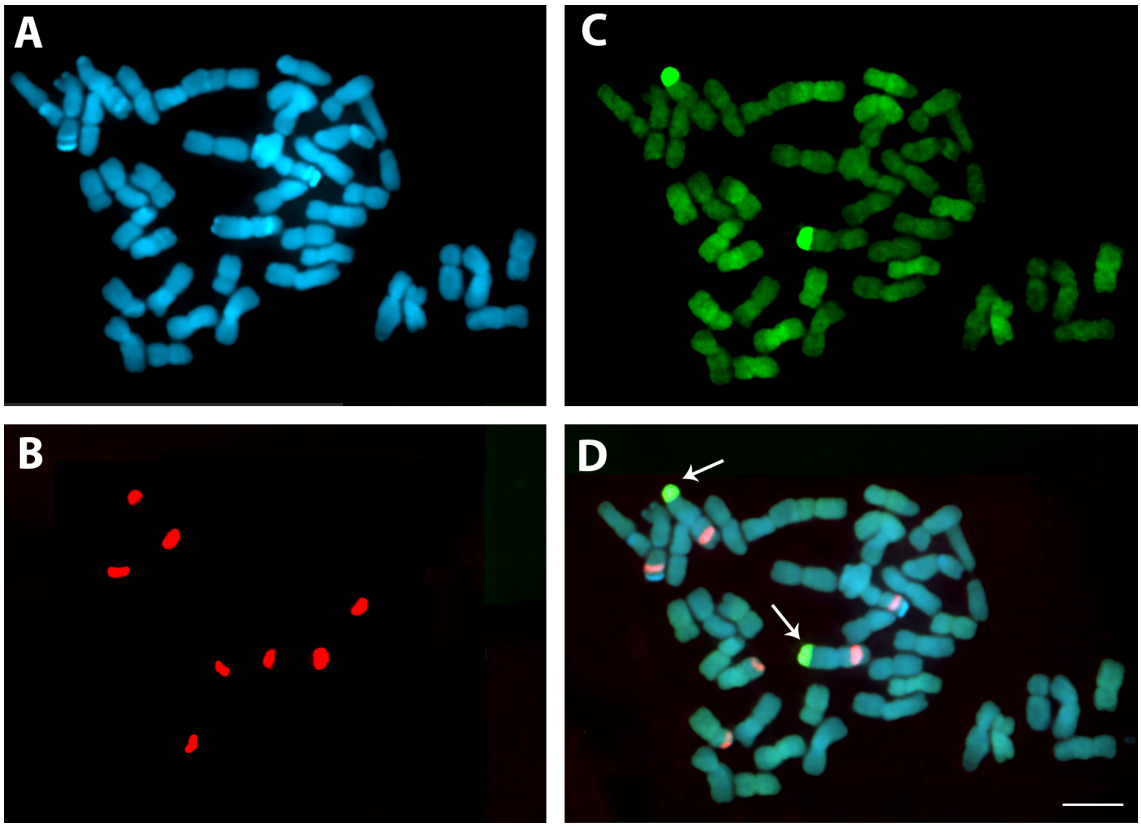


Fig. 3.10: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1164 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization allows detection of *Th. bessarabicum* origin chromosome segment. (C) Hybridization pattern of the *pTa71* clone labeled with biotin-16-dUTP (detected in red) shows the physical location of major 45S rDNA sites in wheat. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

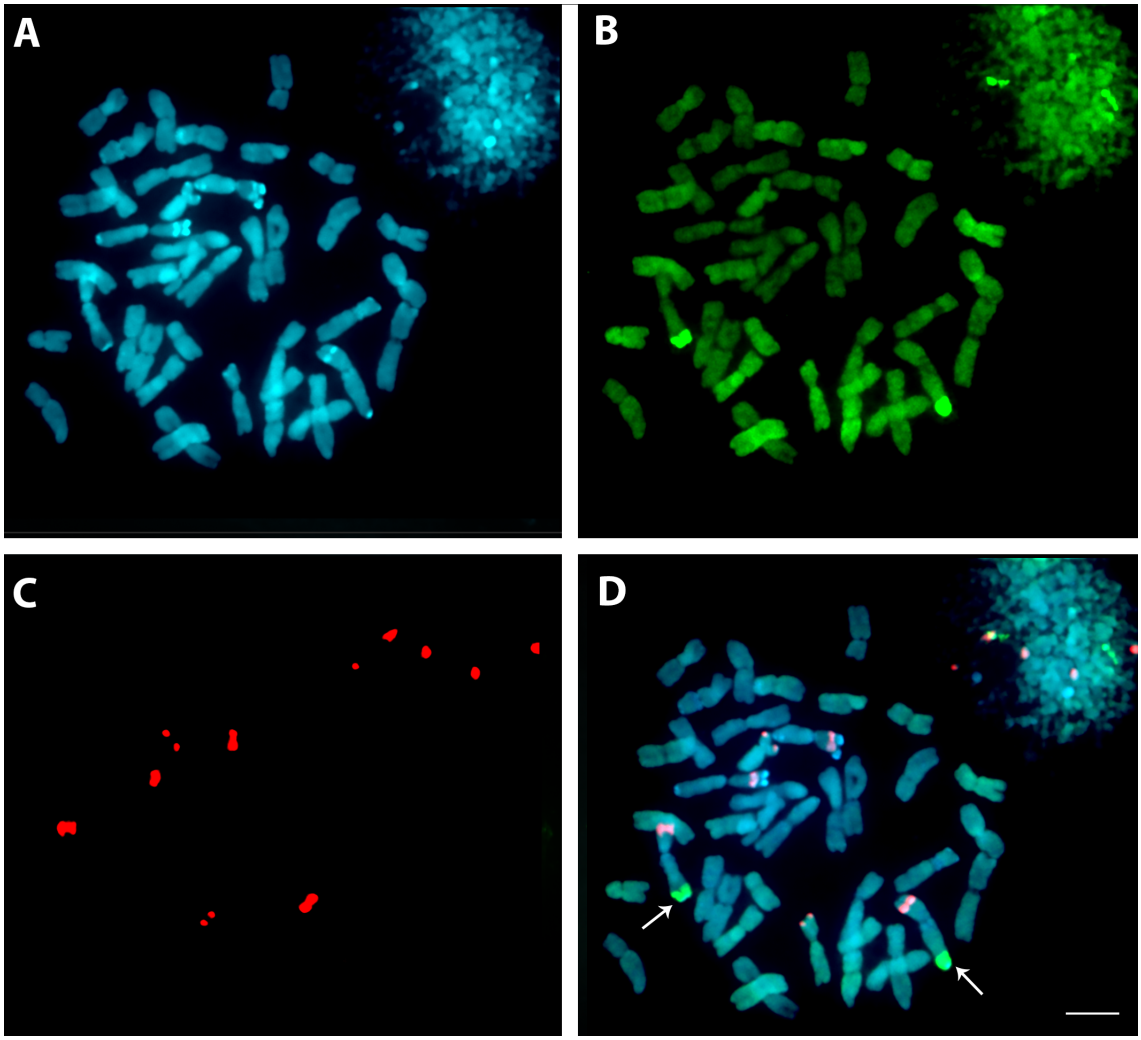


Fig. 3.11: Root tip metaphase chromosome of the wheat-*Th. bessarabicum* introgression line 1164 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization allows detection of *Th. bessarabicum* origin chromosome segment. (C) Hybridization pattern of the *pTa71* clone labeled with biotin-16-dUTP (detected in red) shows the physical location of major 45S rDNA sites in wheat. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

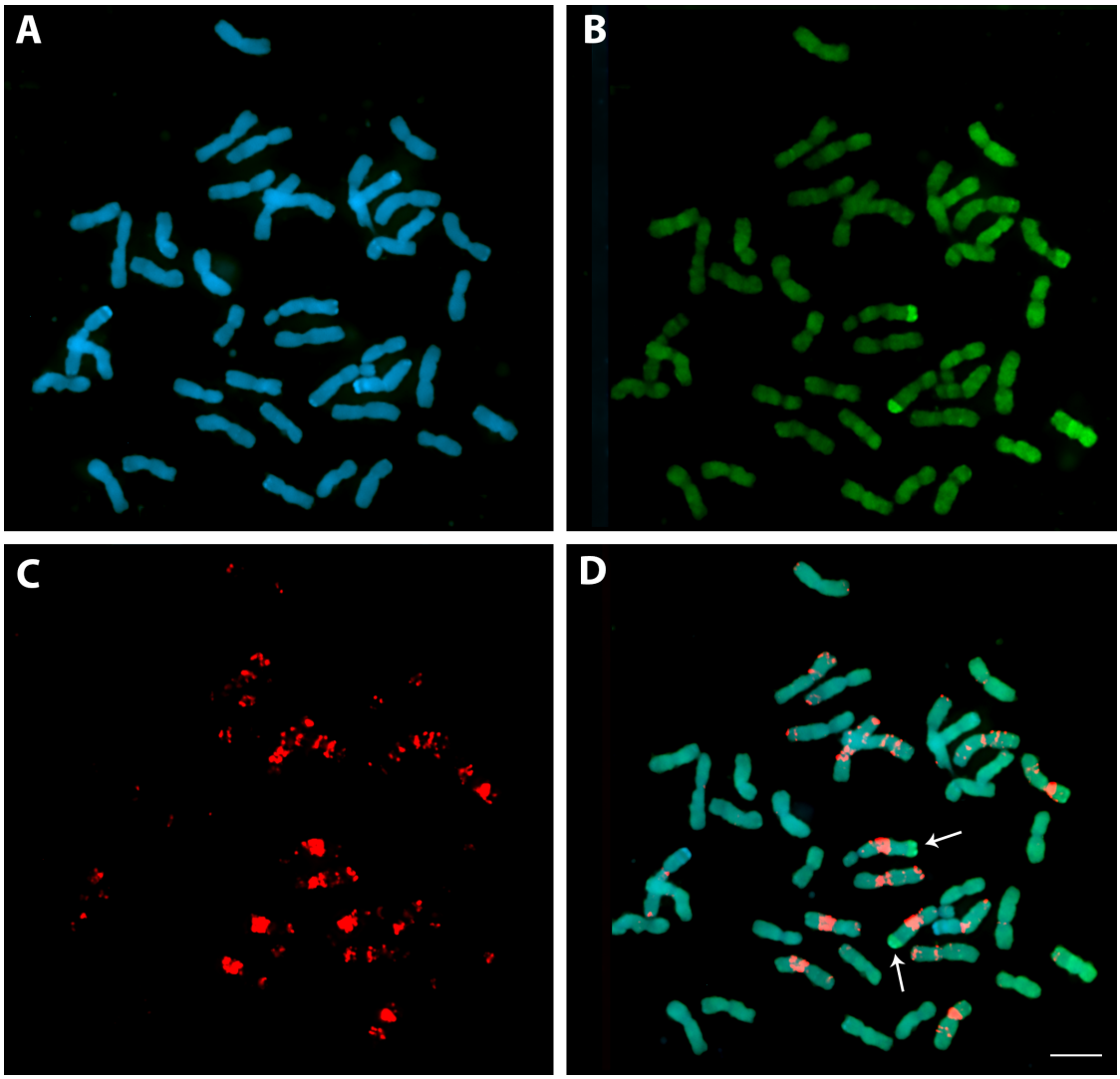


Fig. 3.12: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1164 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization allows detection of *Th. bessarabicum*-origin chromosome segment. (C) Hybridization pattern of the GAA microsatellite sequence labeled with labeled with biotin-16-dUTP (detected in red) that hybridizes preferentially to B-genome and some A-genome chromosomes. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segments.

Scale bar = 10μm.

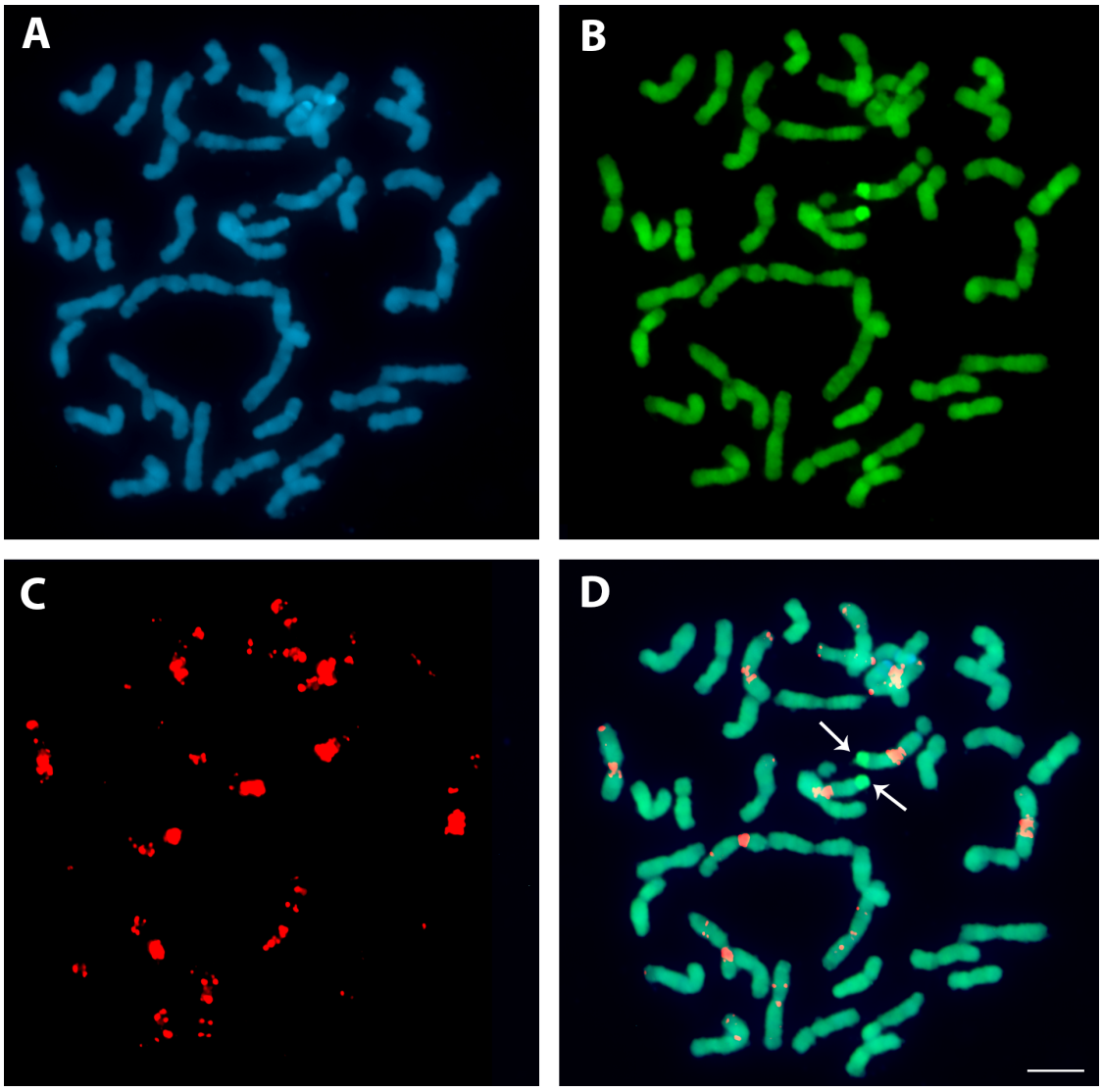


Fig. 3.13: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1164 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization allows detection of *Th. Bessarabicum*-origin chromosome segment. (C) Hybridization pattern of the GAA microsatellite sequence labeled with labeled with biotin-16-dUTP (detected in red) that hybridizes preferentially to B-genome and some A-genome chromosomes. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segments.

Scale bar = 10μm.

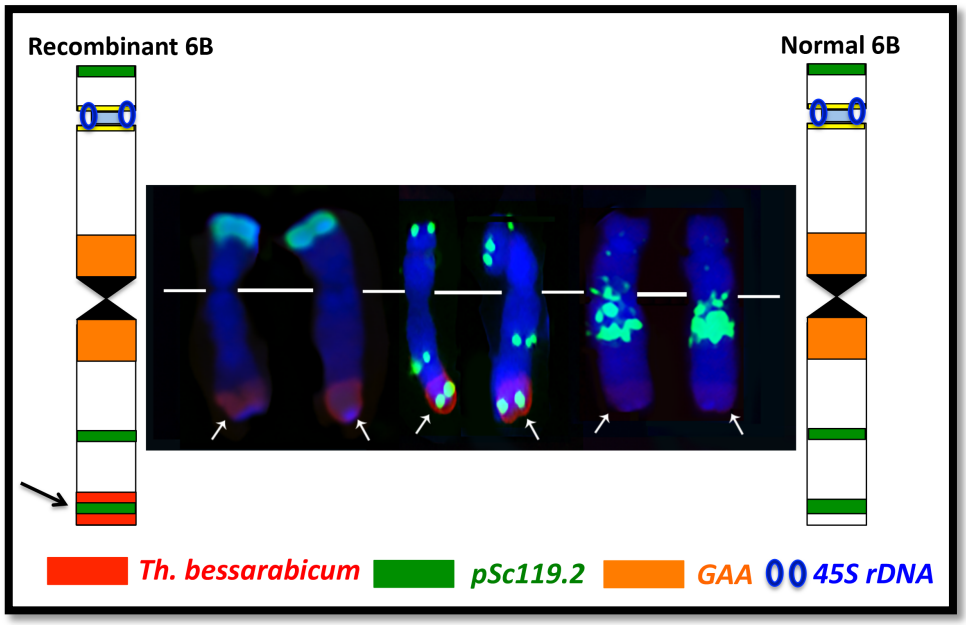


Fig. 3.14: Identification and schematic representation of normal and recombinant wheat chromosome 6B. Sketch of the normal wheat chromosome 6B (right) shows the unique pattern of *pSc119.2* (green), GAA (red) and 45S rDNA (Blue). The recombinant wheat chromosome 6B (left) shows identical banding pattern of *pSc119.2,* GAA and 45S rDNA except the distal band of *pSc119.2* on the long arm is lost due to the translocation with *Th. bessarabicum* chromatin (red).

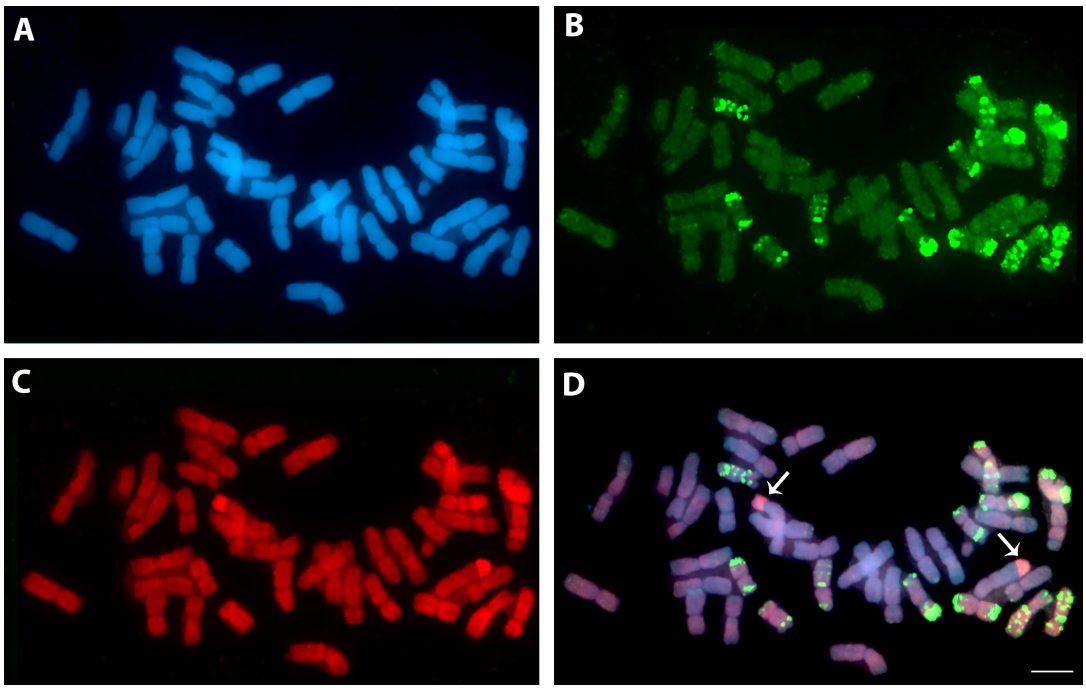


Fig. 3.15: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1168 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes are appearing in blue with DAPI fluorescence (B) Hybridization pattern of the *dpTa1* clone labeled with digoxigenin-11-dUTP (detected in green) which preferentially label D genome of wheat. (C) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with biotin-16-dUTP (detected in red). The genomic *in situ* hybridization allows detection of *Th. bessarabicum*-origin chromosome segment (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale Bar =10μm.

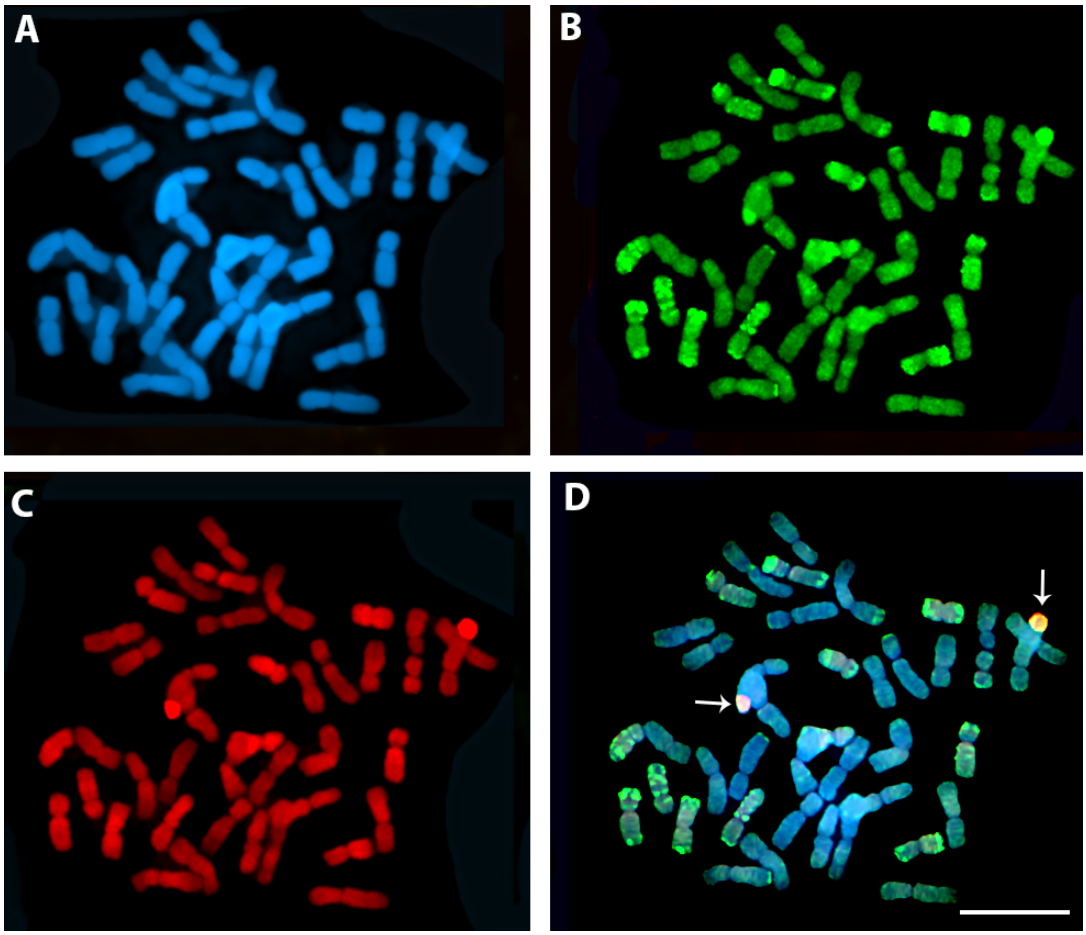


Fig. 3.16: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1168 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes are appearing in blue with DAPI fluorescence (B) Hybridization pattern of the *dpTa1* clone labeled with digoxigenin-11-dUTP (detected in green) which preferentially label D genome of wheat. (C) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with biotin-16-dUTP (detected in red). The genomic *in situ* hybridization allows detection of *Th. bessarabicum*-origin chromosome segment (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale Bar =10μm.

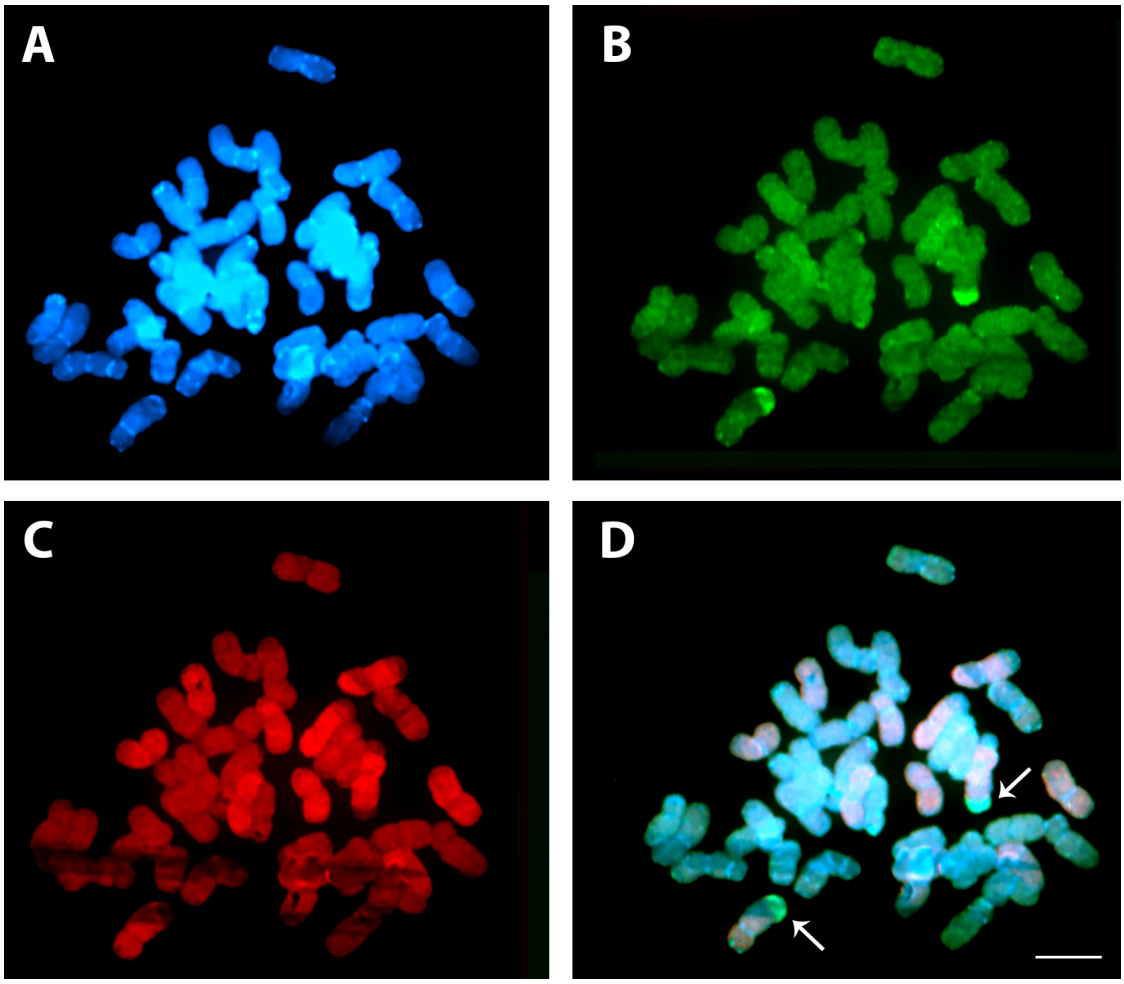


Fig. 3.17: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1168 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization allows detection of *Th. bessarabicum* origin chromosome segment. (C) *In situ* hybridization of the total genomic DNA from *T. monococcum* labeled with biotin-16-dUTP (detected in red). The genomic *in situ* hybridization labeled seven pairs of A-genome. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale Bar =10μm.

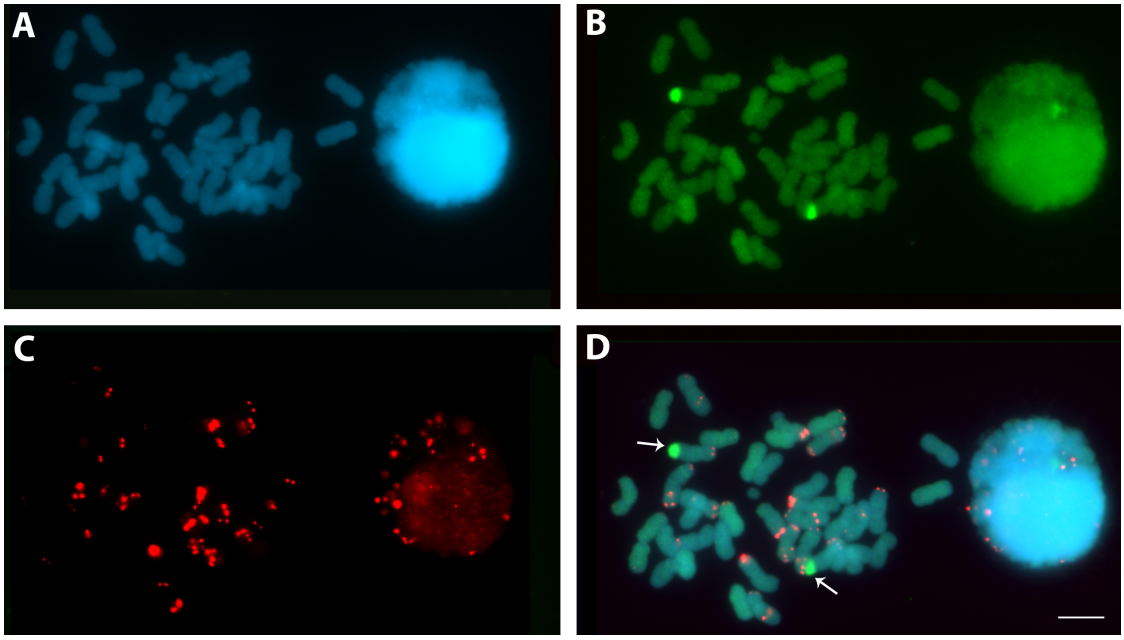


Fig. 3.18: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1168 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green), which allows detection of *Th. bessarabicum* origin chromosome segment. (C) Hybridization pattern of the *pSc119.2* DNA sequence labeled with biotin-16-dUTP (detected in red) that hybridize preferentially to B-genome and some A-genome chromosomes. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

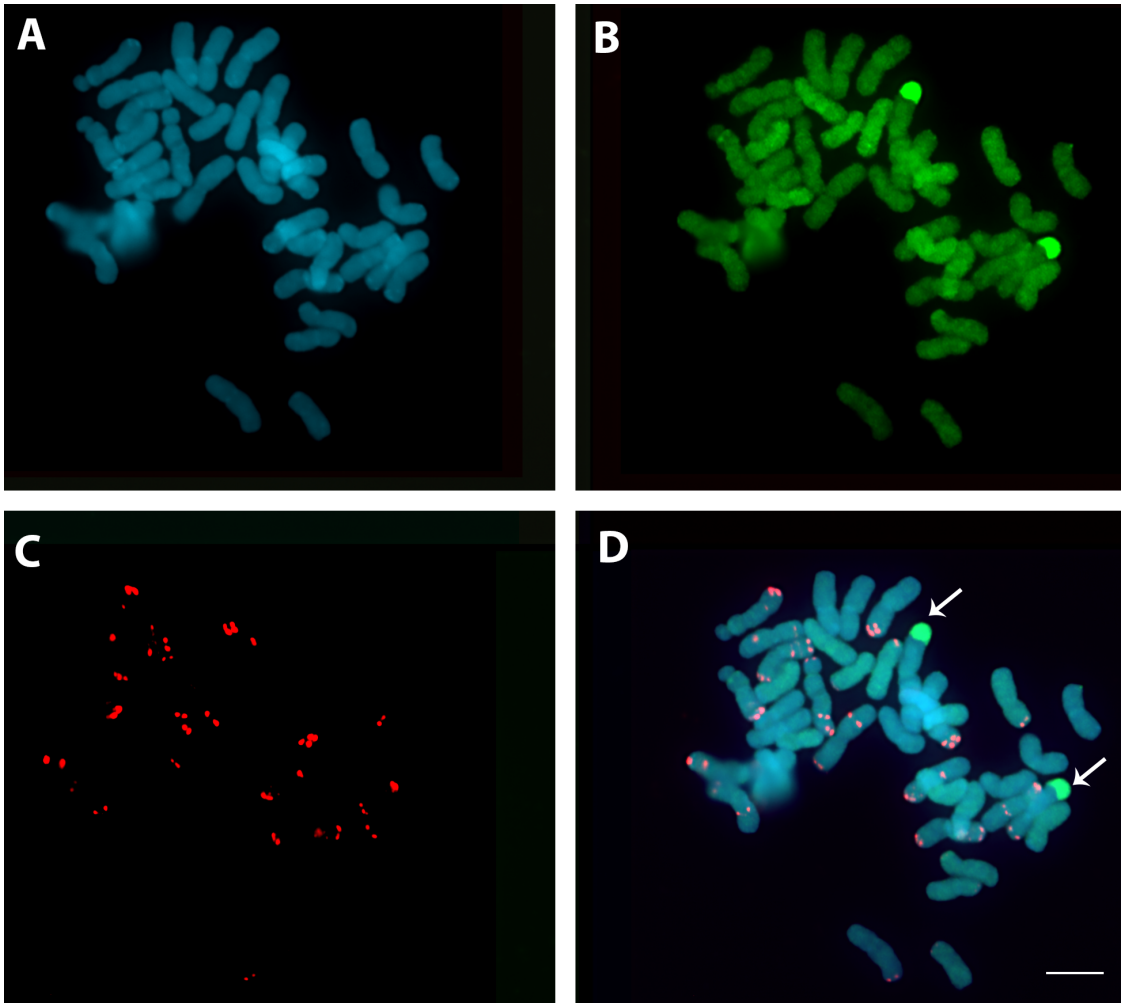


Fig. 3.19: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1168 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green), which allows detection of *Th. bessarabicum* origin chromosome segment. (C) Hybridization pattern of the *pSc119.2* DNA sequence labeled with biotin-16-dUTP (detected in red) that hybridize preferentially to B-genome and some A-genome chromosomes. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

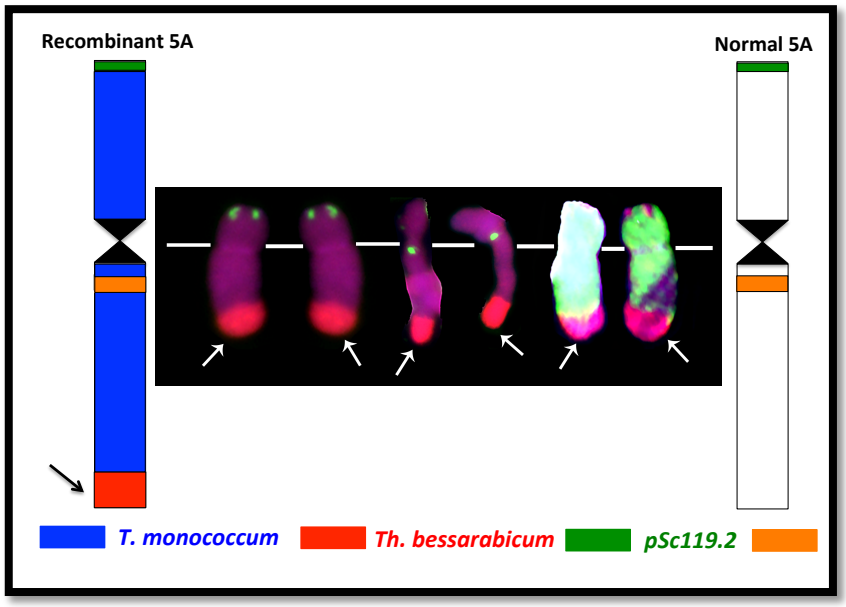


Fig. 3.20: Identification and schematic representation of normal and recombinant wheat chromosome 5A. Sketch of the normal wheat chromosome 5A (left) shows the unique pattern of *pSc119.2* (green), GAA (yellow). The recombinant wheat chromosome 5A (right) shows identical banding pattern of *pSc119.2* and GAA, except strong signal of *Th. bessarabicum* chromatin (red) on the long arm is due translocation with *Th. bessarabicum* chromatin (red) and strongly labelled genomic DNA *T. monococcum* (blue).

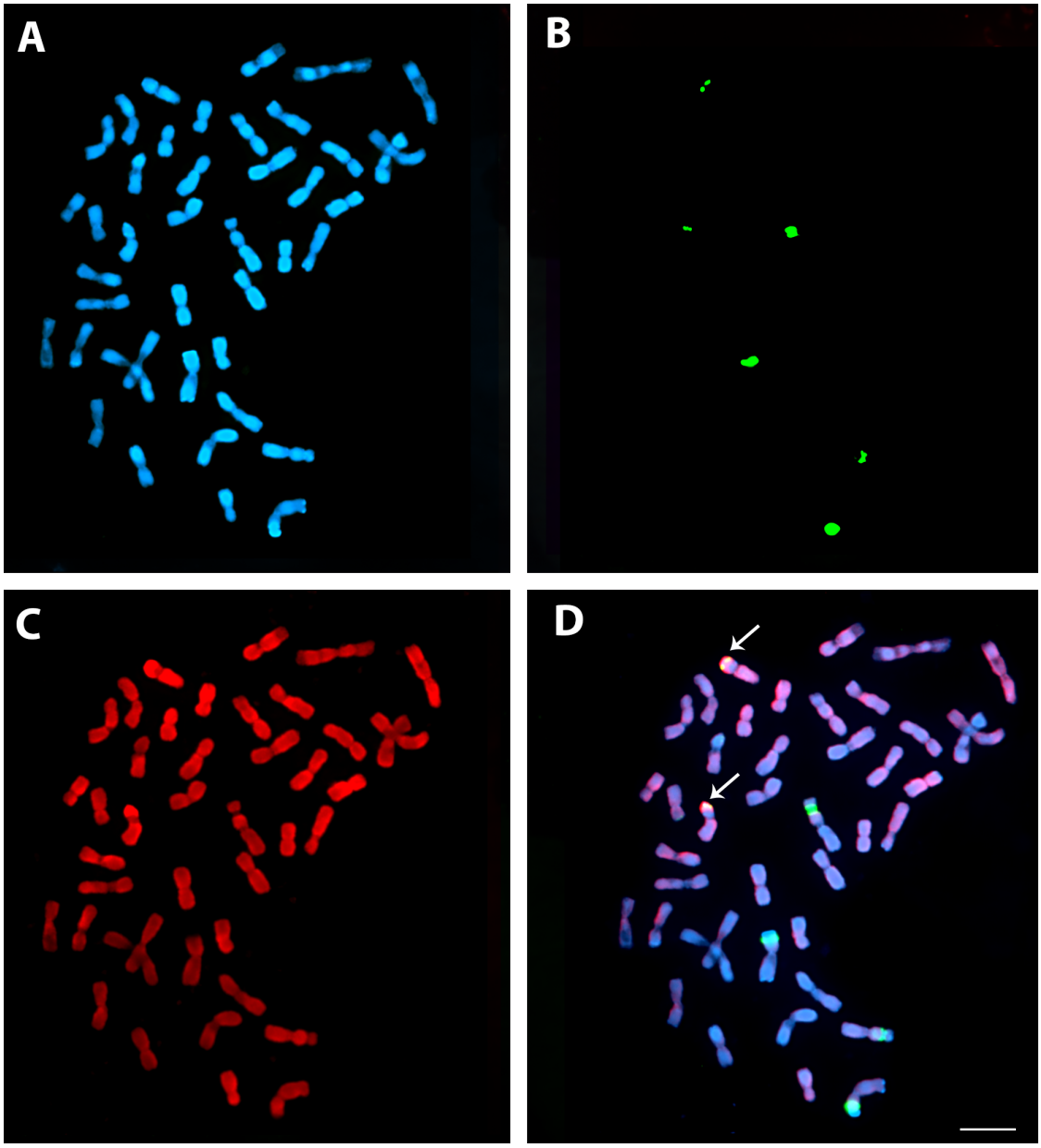


Fig. 3.21: Root tip metaphase chromosomes of the *wheat-Th. bessarabicum* introgression line 1172 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) Hybridization pattern of the *pTa71* clone labeled with digoxigenin-11-dUTP (detected in green) showing the physical location of major 45S rDNA sites in wheat. (C) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with biotin-16-dUTP (detected in red). The genomic *in situ* hybridization allows detection of *Th. bessarabicum* origin chromatin segment. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale Bar =10μm.

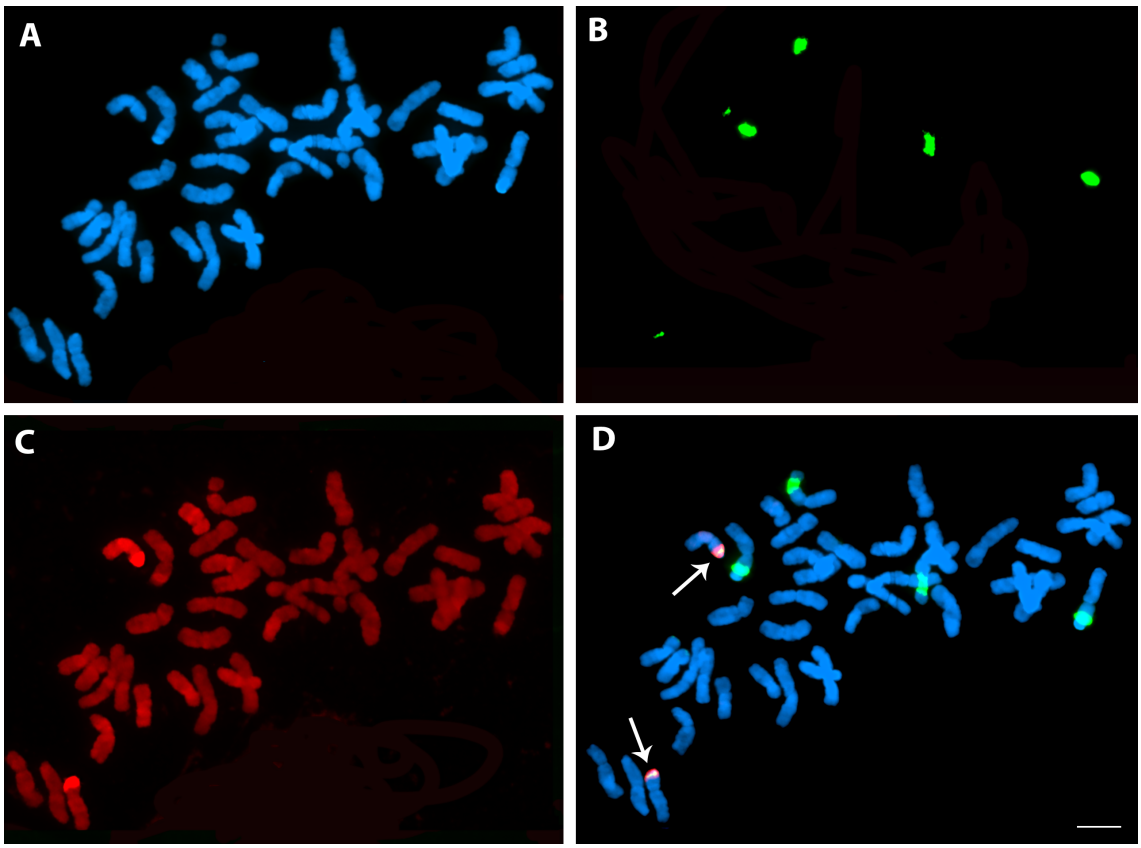


Fig. 3.22: Root tip metaphase chromosomes of the *wheat-Th. bessarabicum* introgression line 1172 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) Hybridization pattern of the *pTa71* clone labeled with digoxigenin-11-dUTP (detected in green) showing the physical location of major 45S rDNA sites in wheat. (C) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with biotin-16-dUTP (detected in red). The genomic *in situ* hybridization allows detection of *Th. bessarabicum*-origin chromatin segment. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

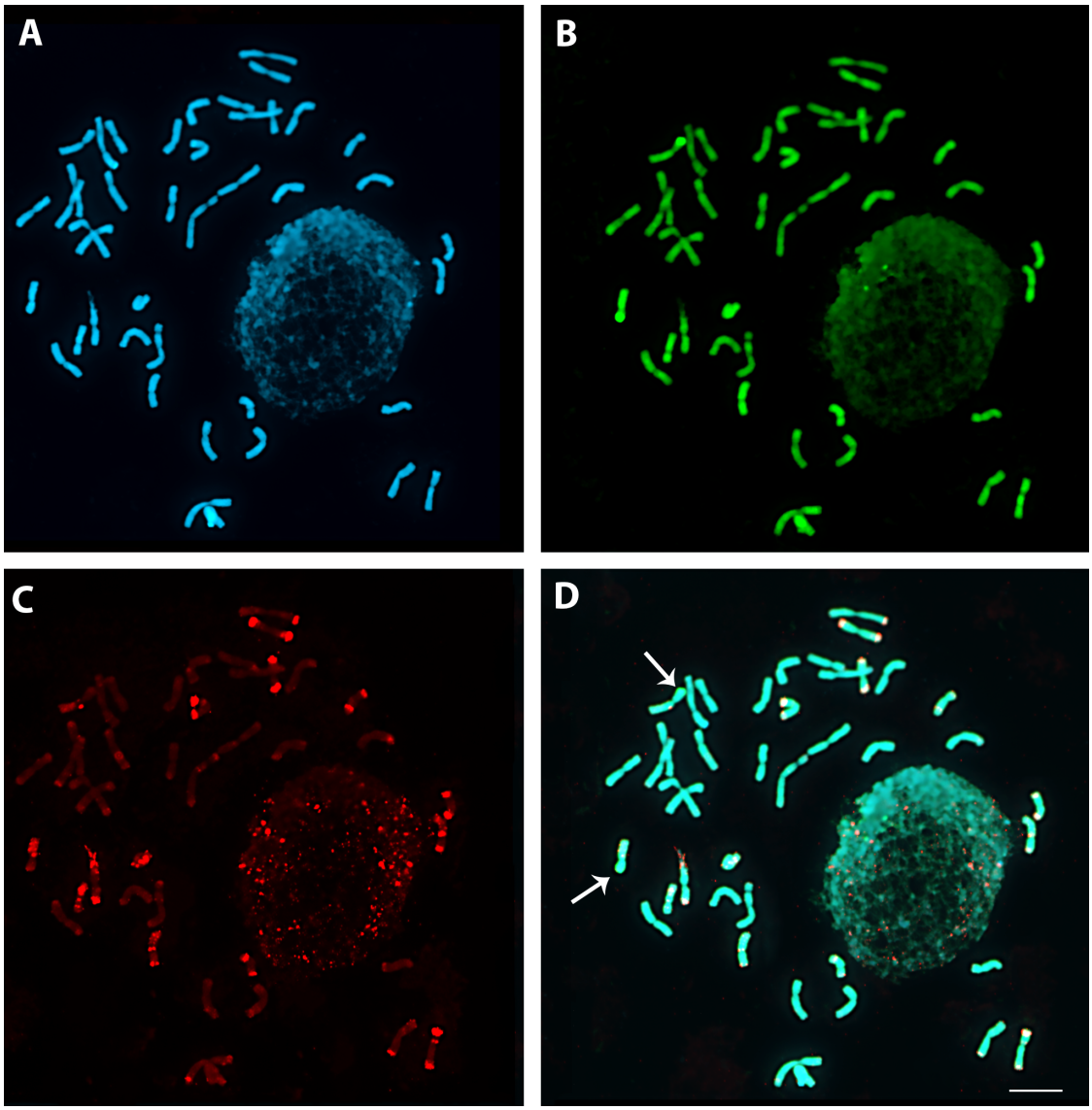


Fig. 3.23: Root tip metaphase chromosomes of the *wheat-Th. bessarabicum* introgression line 1172 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization allows detection of *Th. bessarabicum*-origin chromosome segment. (C) Hybridization pattern of the *dpTa1* clone labeled with biotin-16-dUTP (detected in red) which preferentially labels D-genome of wheat. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

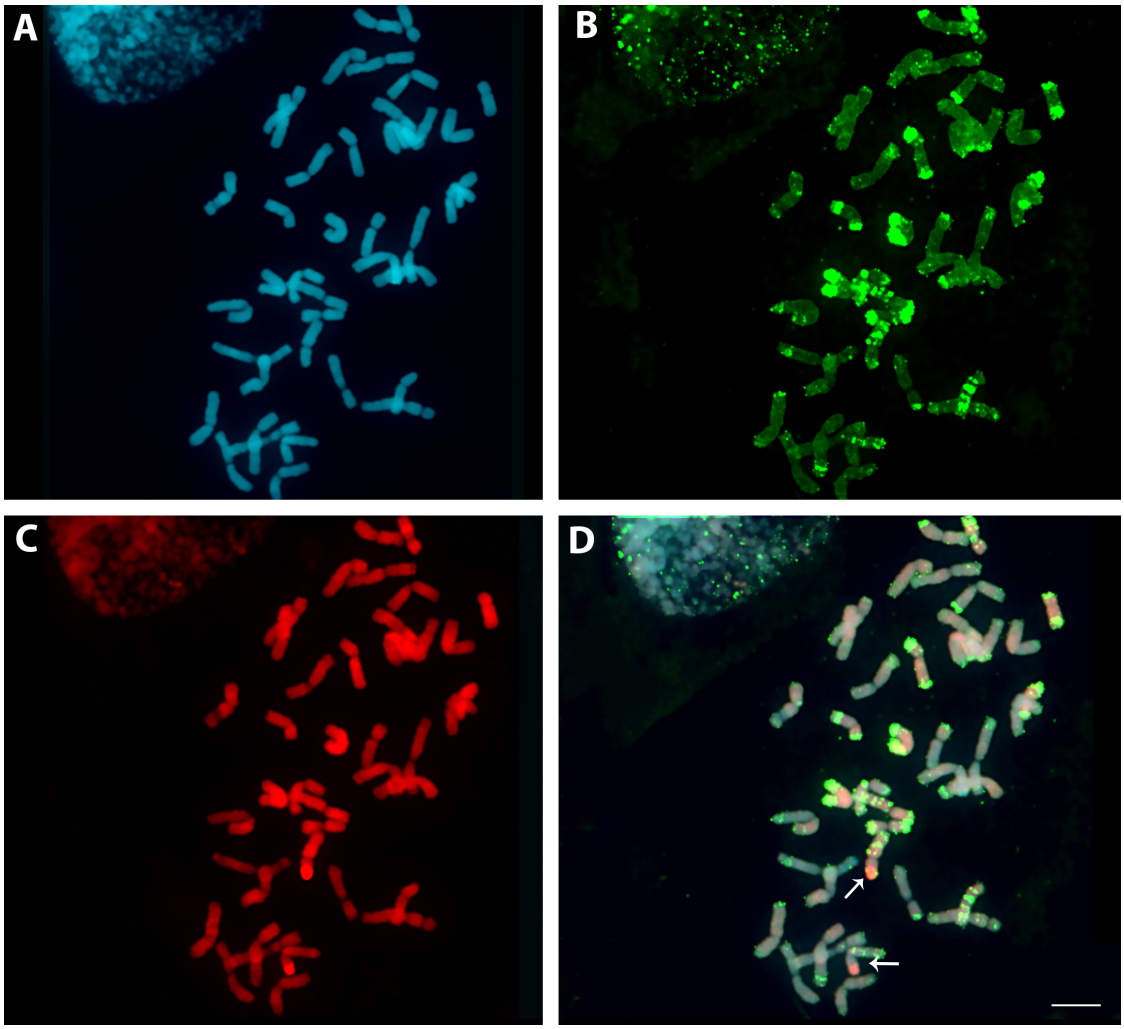


Fig. 3.24: Root tip metaphase chromosomes of the *wheat-Th. bessarabicum* introgression line 1172 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence (B) Hybridization pattern of the *dpTa1* clone labeled with digoxigenin-11-dUTP (detected in green) which preferentially labels D-genome of wheat. (C) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with biotin-16-dUTP (detected in red). The genomic *in situ* hybridization allows detection of *Th. bessarabicum*-origin chromosome segment (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

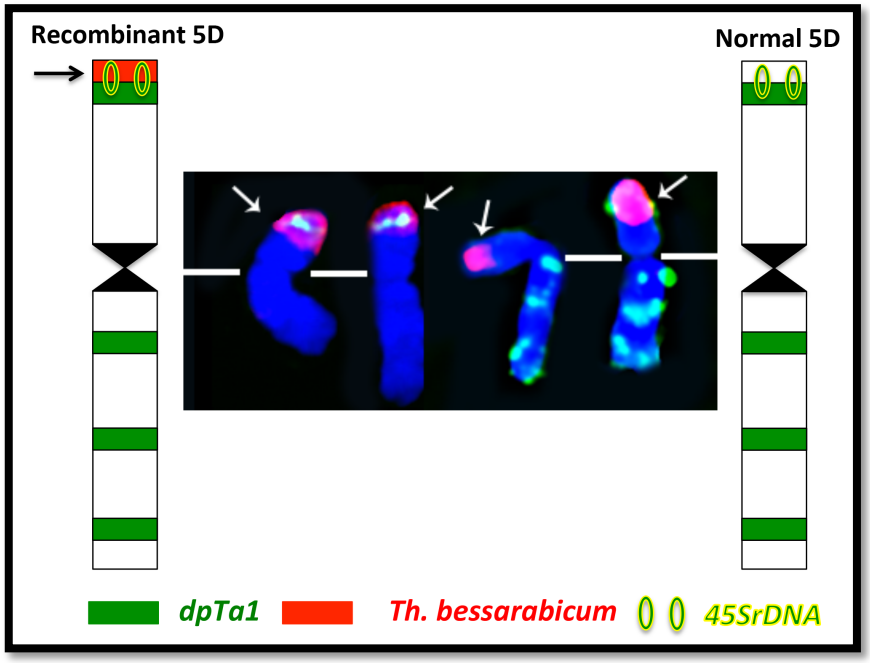


Fig. 3.25: Identification and schematic representation of normal and recombinant wheat chromosome 5D. Sketch of the normal wheat chromosome 5D (left) shows the unique pattern of *dpTa1* (green), 45S rDNA (orange-green). The recombinant wheat chromosome 5D (right) shows identical banding pattern of *dpTa1* (green) and 45S rDNA (orange-green) except strong signal of *Th. bessarabicum* chromatin (red) on the short arm is due translocation with *Th. bessarabicum* chromatin (red).

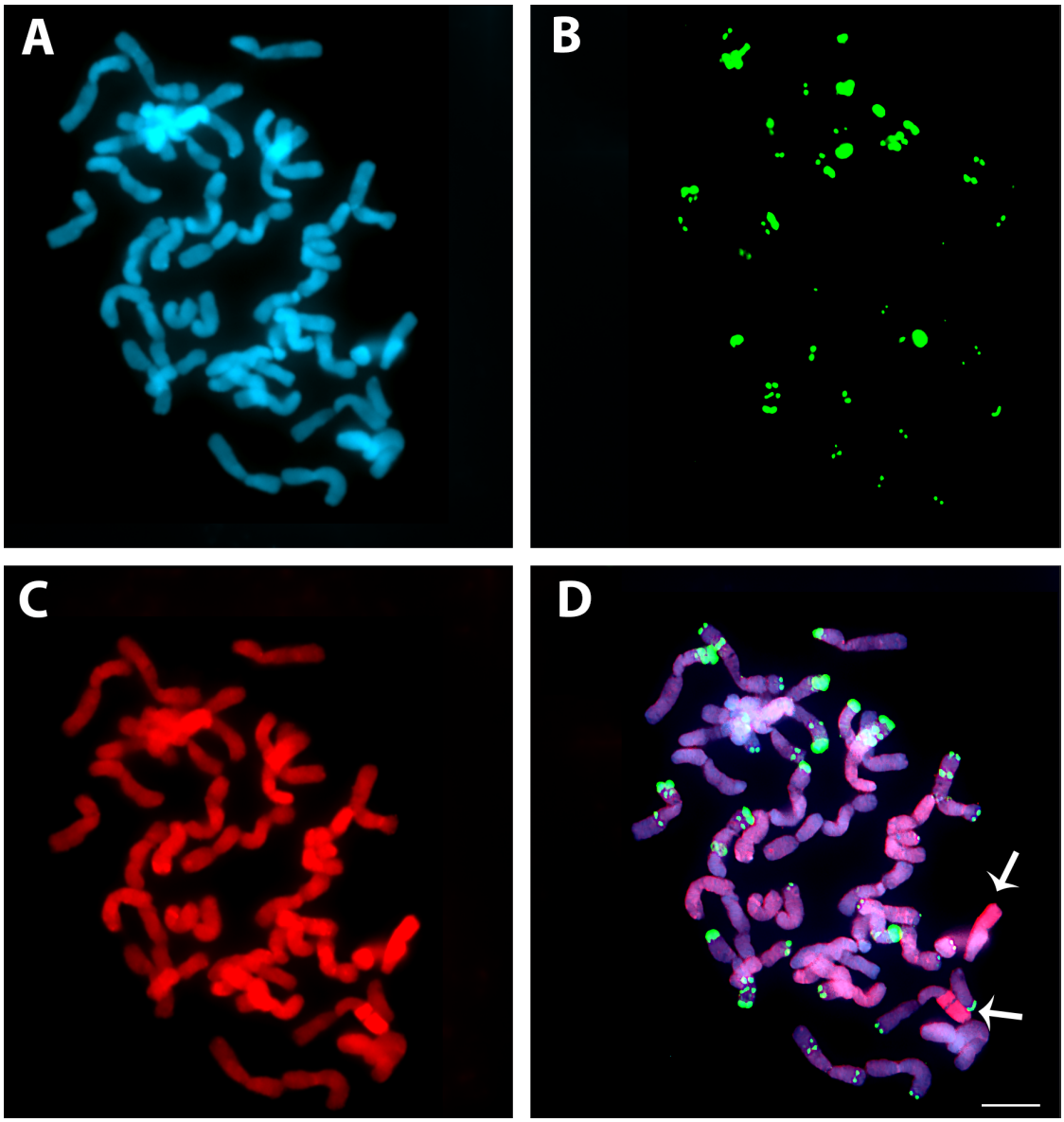


Fig. 3.26: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1176 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) Hybridization pattern of the *pSc119.2* DNA sequence labeled with digoxigenin -1-dUTP (detected in green) that hybridize preferentially to b and some A-genome chromosomes. (C) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with biotin-16-dUTP (detected in red). The genomic *in situ* hybridization allows detection of *Th. bessarabicum*-origin chromosome segment. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segments. Scale bar =10μm.

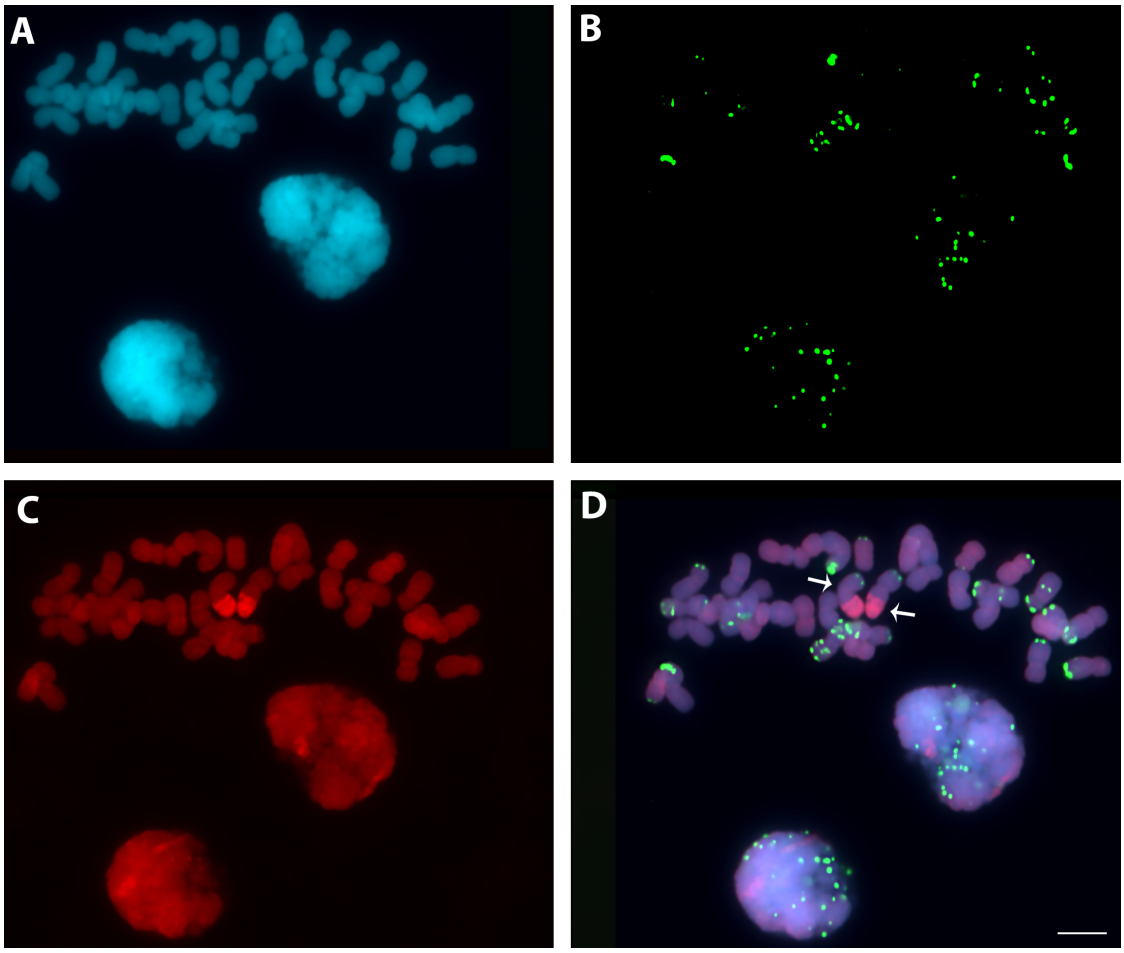


Fig. 3.27: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1176 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) Hybridization pattern of the *pSc119.2* DNA sequence labeled with digoxigenin -11-dUTP (detected in green) that hybridize preferentially to b and some A-genome chromosomes. (C) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with biotin-16-dUTP (detected in red). The genomic *in situ* hybridization allows detection of *Th. bessarabicum* origin chromosome segment. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segments. Scale bar =10μm.

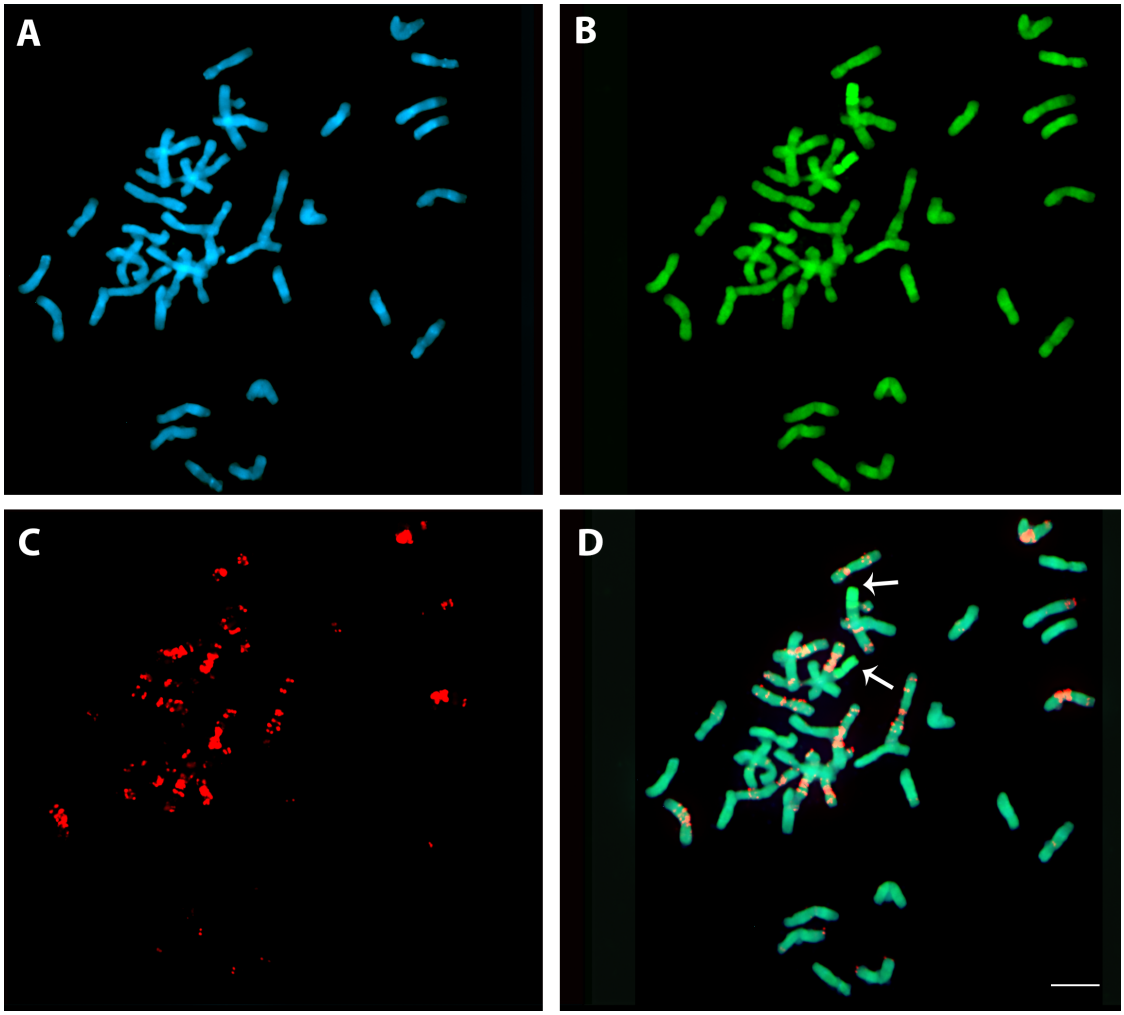


Fig. 3.28: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1176 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization allows the detection of *Th. bessarabicum* origin chromosome segment. (C) Hybridization pattern of the GAA microsatellite sequence labeled with labeled with biotin-16-dUTP (detected in red) that hybridizes preferentially to B-genome and some A-genome chromosomes. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segments.

Scale bar =10μm.

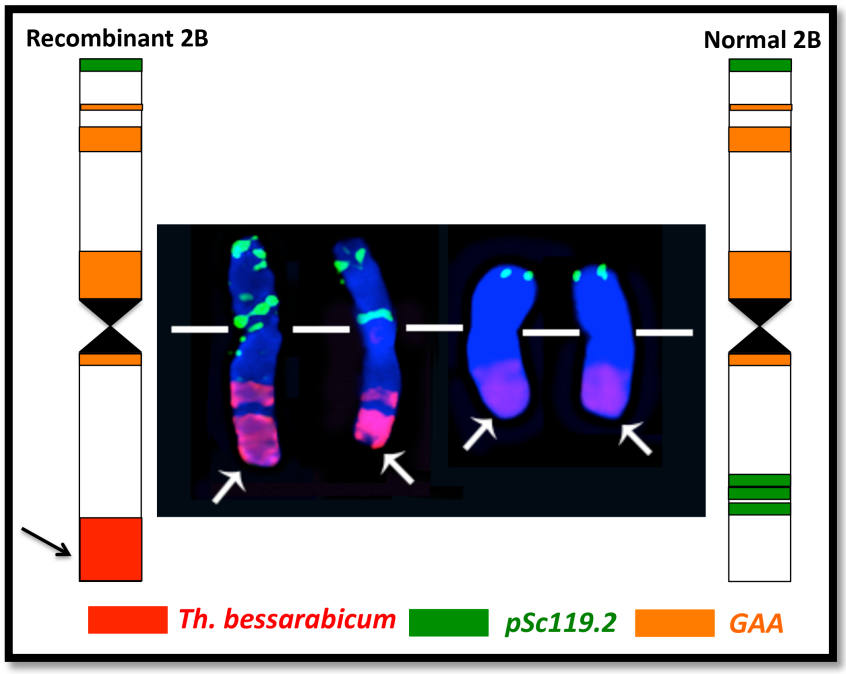


Fig. 3.29: Identification and schematic representation of normal and recombinant wheat chromosome 2B. Sketch of the normal wheat chromosome 2B (left) shows the unique pattern of *pSc119.2* (green), GAA (yellow). The recombinant wheat chromosome 2B (right) shows identical banding pattern of *pSc119.2* and GAA, except the two intercalary band of *pSc119.2* on the long arm is lost due to translocation with *Th. bessarabicum* chromatin (red).

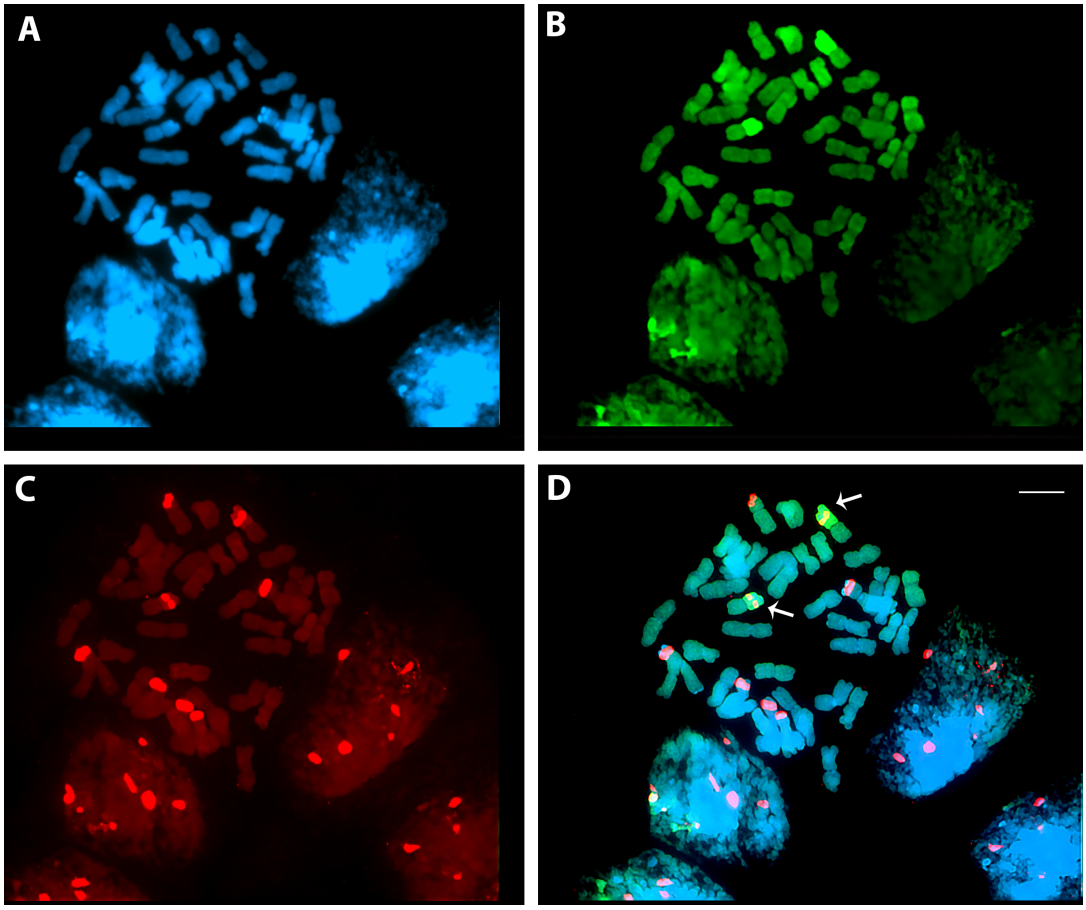


Fig. 3.30: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1180 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization allows detection of *Th. bessarabicum*-origin chromatin segment. (C) The hybridization pattern of the *pTa71* clone labeled with biotin-16-dUTP (detected in red) shows the physical location of major 45S rDNA sites in wheat. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale Bar =10μm.

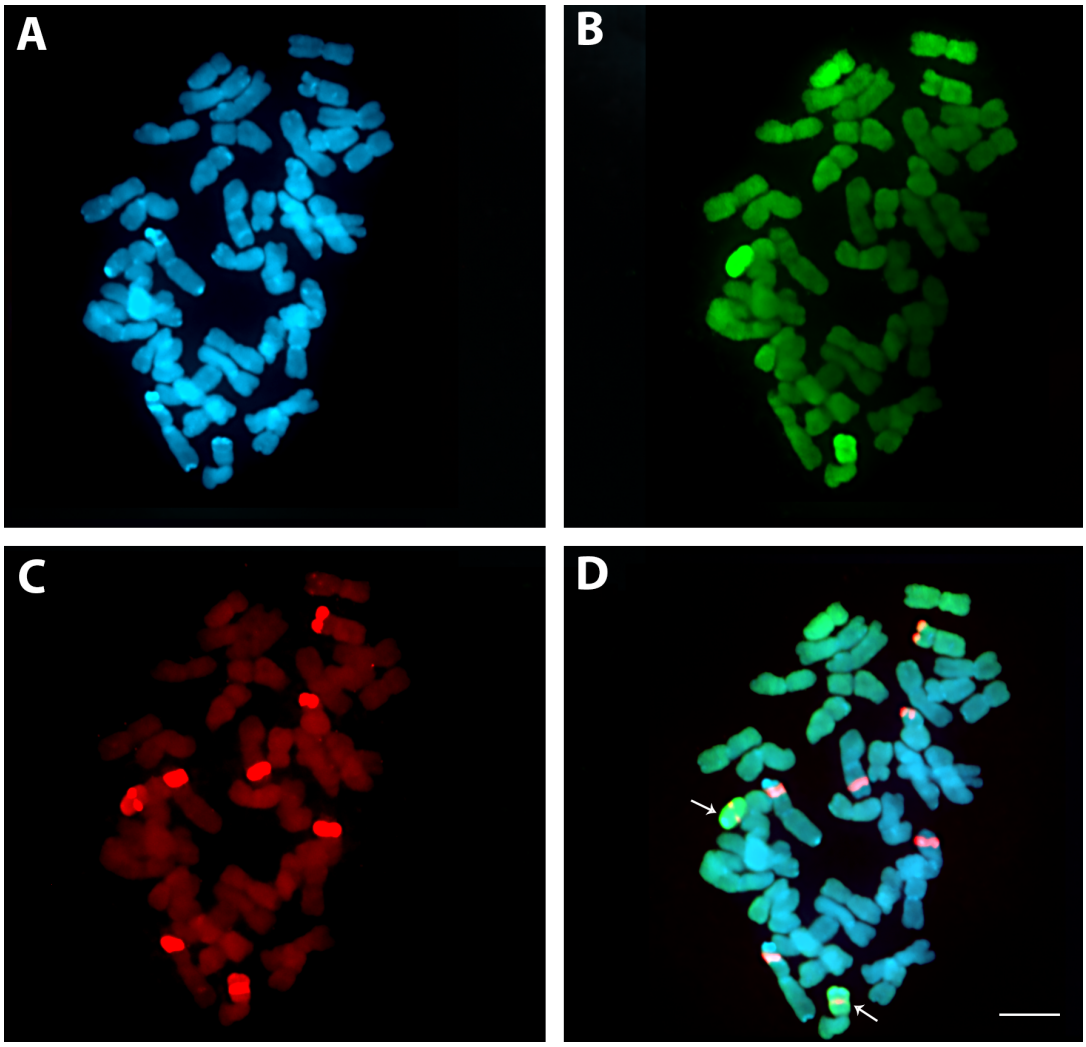


Fig. 3.31: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1180 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization allows detection of *Th. bessarabicum*-origin chromatin segment. (C) The hybridization pattern of the *pTa71* clone labeled with biotin-16-dUTP (detected in red) shows the physical location of major 45S rDNA sites in wheat. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale Bar =10μm.

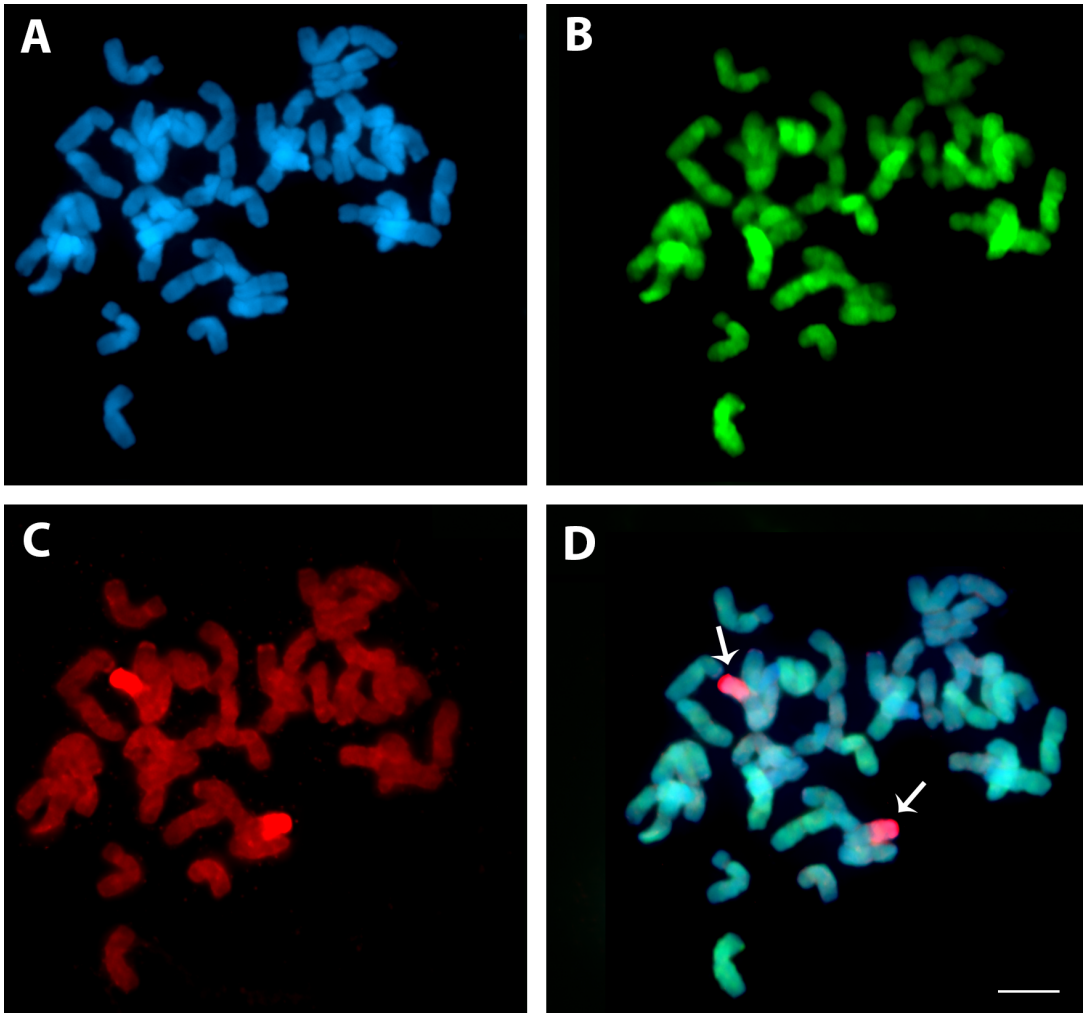


Fig. 3.32: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1180 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *T. monococcum* labeled with digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization labeled seven pairs of A-genome. (C) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with biotin-16-dUTP (detected in red). The genomic *in situ* hybridization allows detection of *Th. bessarabicum* origin chromosome segment. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale Bar =10μm.

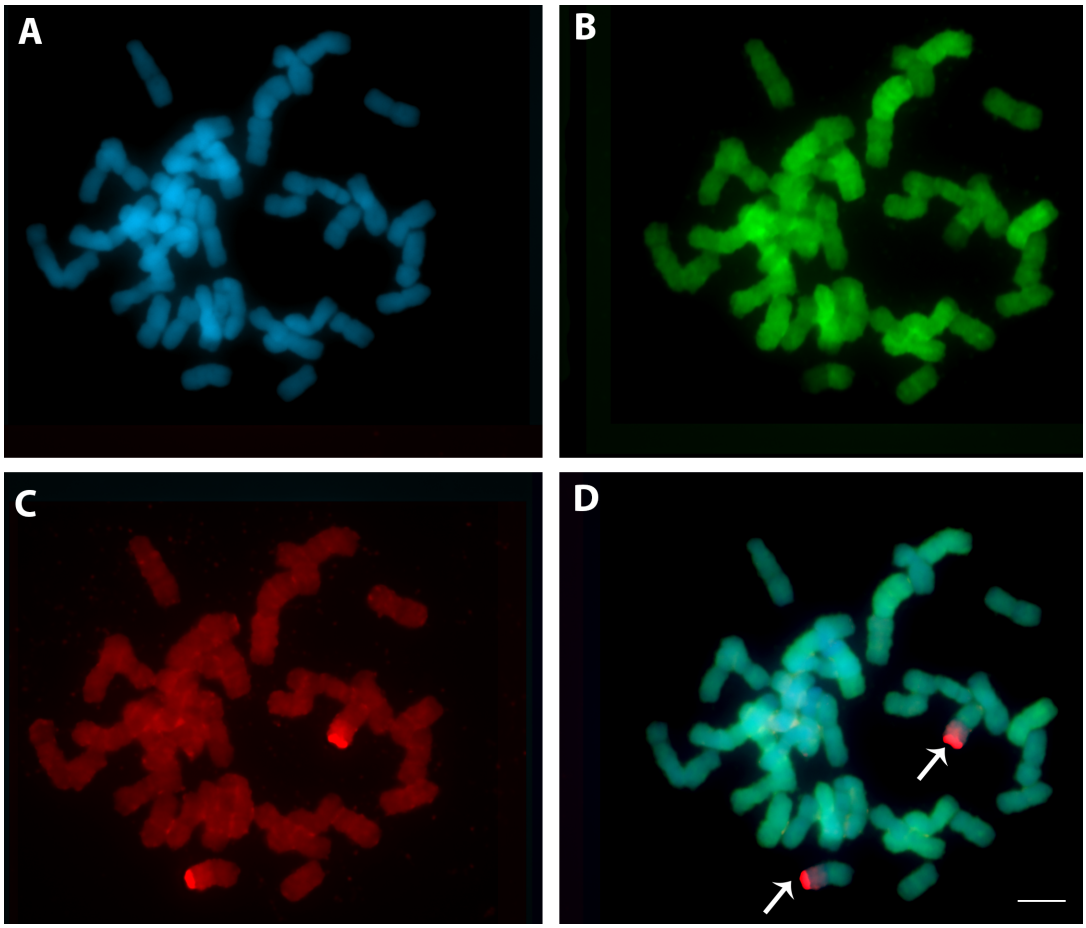


Fig. 3.33: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1180 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) *In situ* hybridization of the total genomic DNA from *T. monococcum* labeled with digoxigenin-11-dUTP (detected in green). The genomic *in situ* hybridization labeled seven pairs of A-genome. (C) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with biotin-16-dUTP (detected in red). The genomic *in situ* hybridization allows detection of *Th. bessarabicum* origin chromosome segment. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale Bar =10μm.

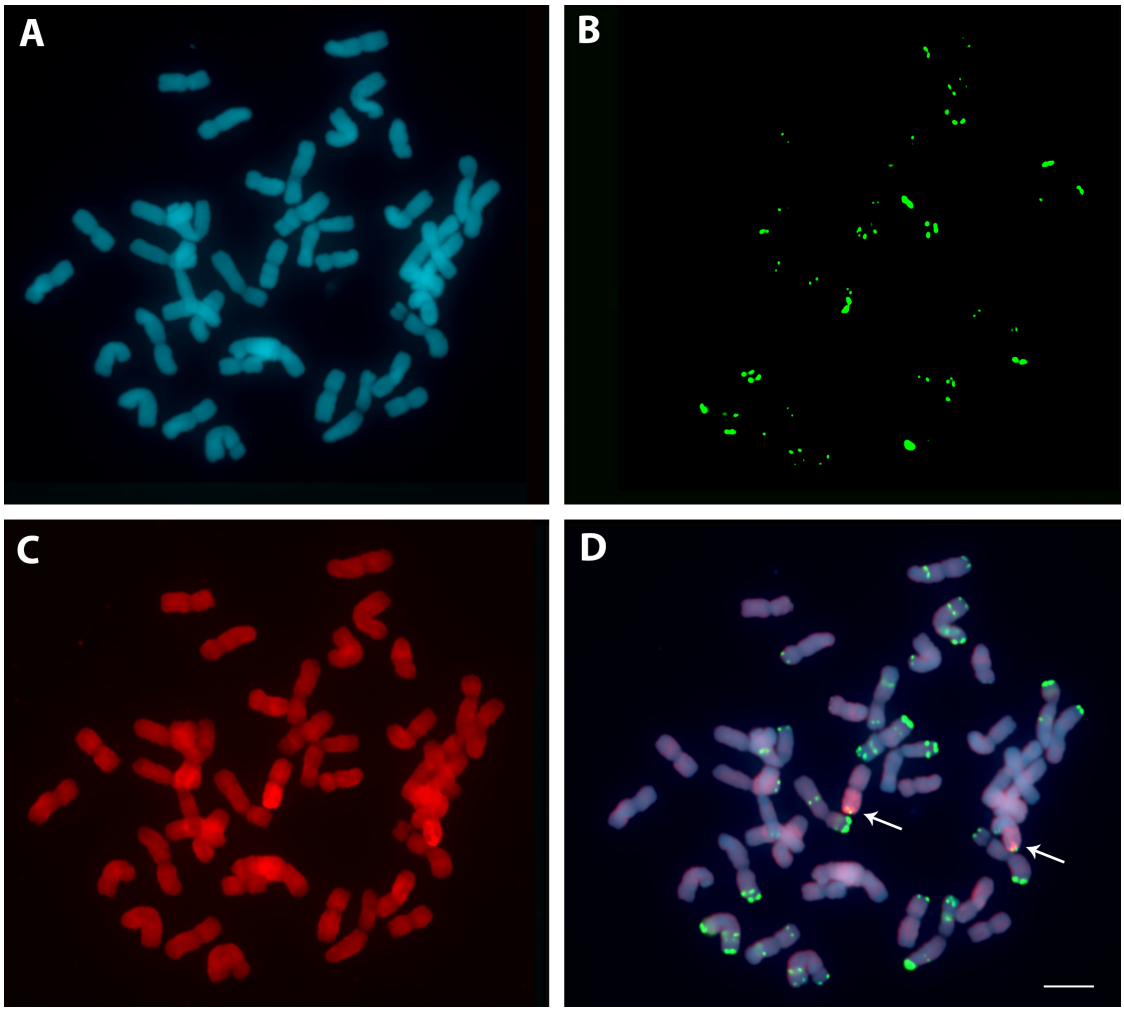


Fig. 3.34: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1180 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) Hybridization pattern of the *pSc119.2* DNA sequence labeled with digoxigenin-11-dUTP (detected in green) that hybridize preferentially to B-genome and some A-genome chromosomes. (C) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with biotin-16-dUTP (detected in red). The genomic *in situ* hybridization allows the detection of *Th. bessarabicum* origin chromatin segment. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale Bar =10μm.

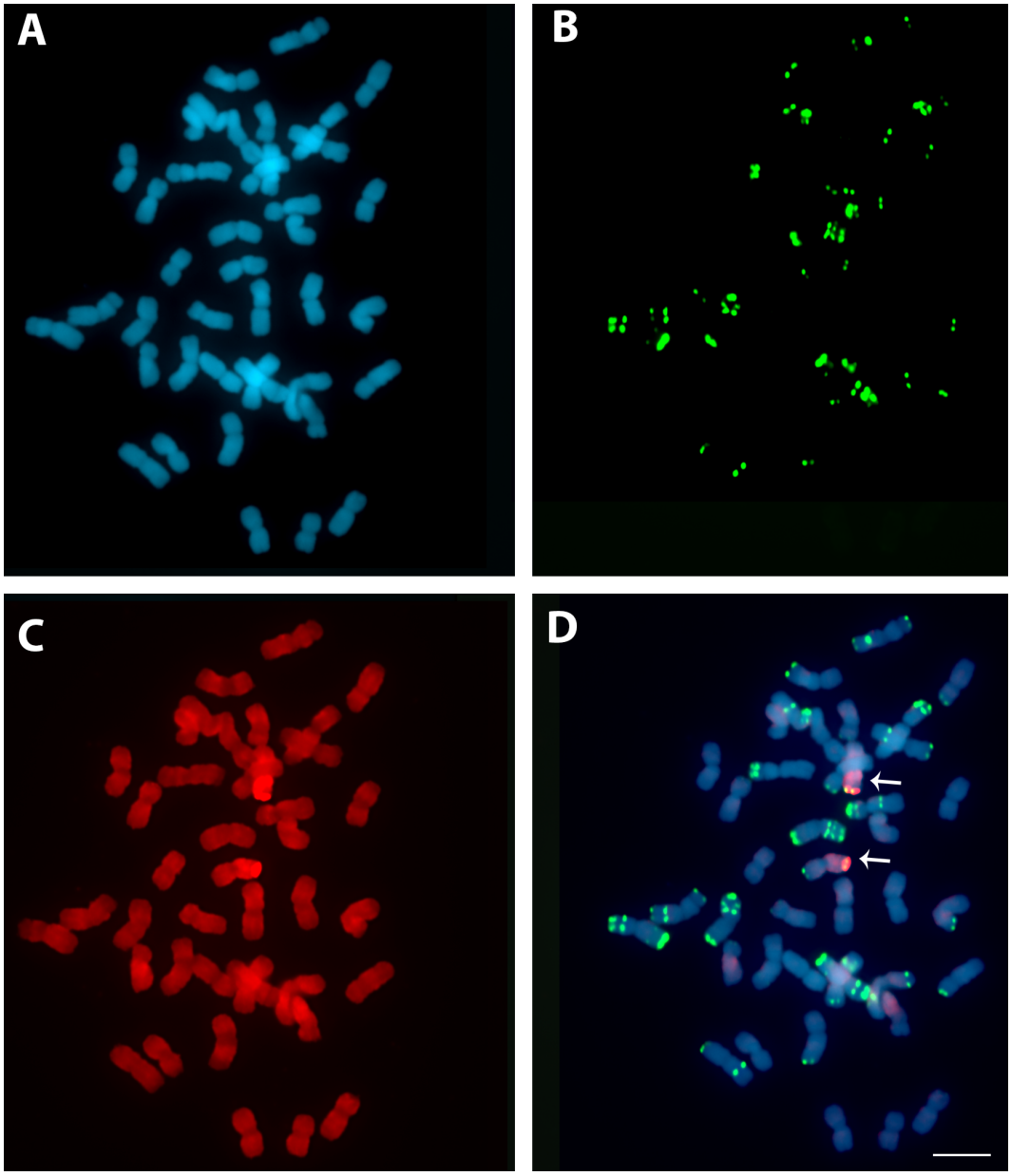


Fig. 3.35: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* introgression line 1180 (2*n*=42) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence. (B) Hybridization pattern of the *pSc119.2* DNA sequence labeled with digoxigenin-11-dUTP (detected in green) that hybridize preferentially to B-genome and some A-genome chromosomes. (C) *In situ* hybridization of the total genomic DNA from *Th. bessarabicum* labeled with biotin-16-dUTP (detected in red). The genomic *in situ* hybridization allows the detection of *Th. bessarabicum* origin chromatin segment. (D) Overlay of A, B and C images. Arrows indicate alien chromatin segment. Scale bar =10μm.

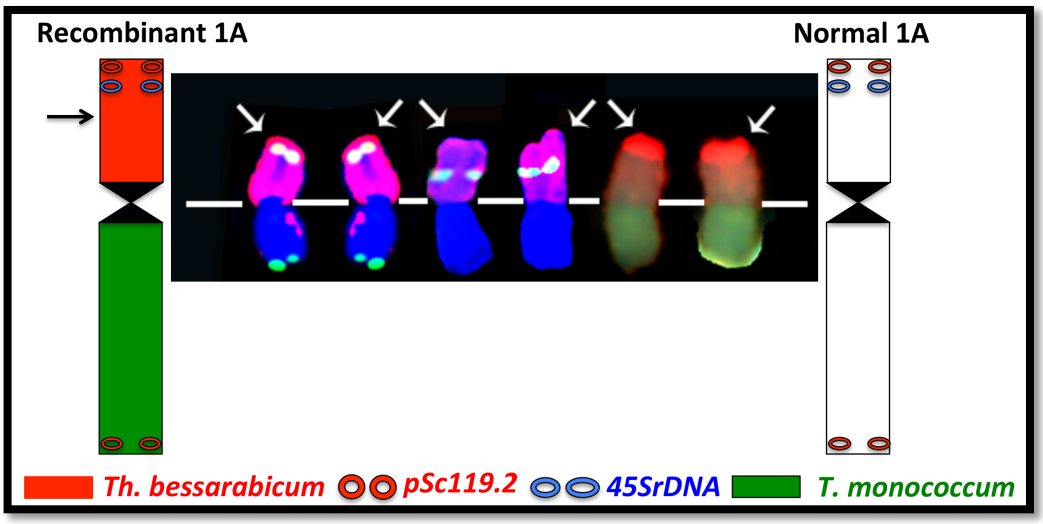


Fig. 3.36: Identification and schematic representation of normal and recombinant wheat chromosome 1A. Sketch of the normal wheat chromosome 1A (left) shows the unique pattern of *pSc119.2* (red), 45S rDNA (blue). The recombinant wheat chromosome 1A (right) shows identical banding pattern of *pSc119.2* and 45S rDNA, except the short arm of the normal wheat chromosome 1AS is replaced by short of the *Th. bessarabicum* chromatin 1JS (red) and *T. monococcum* DNA strongly labelled long arm of the wheat chromosome 1AL.

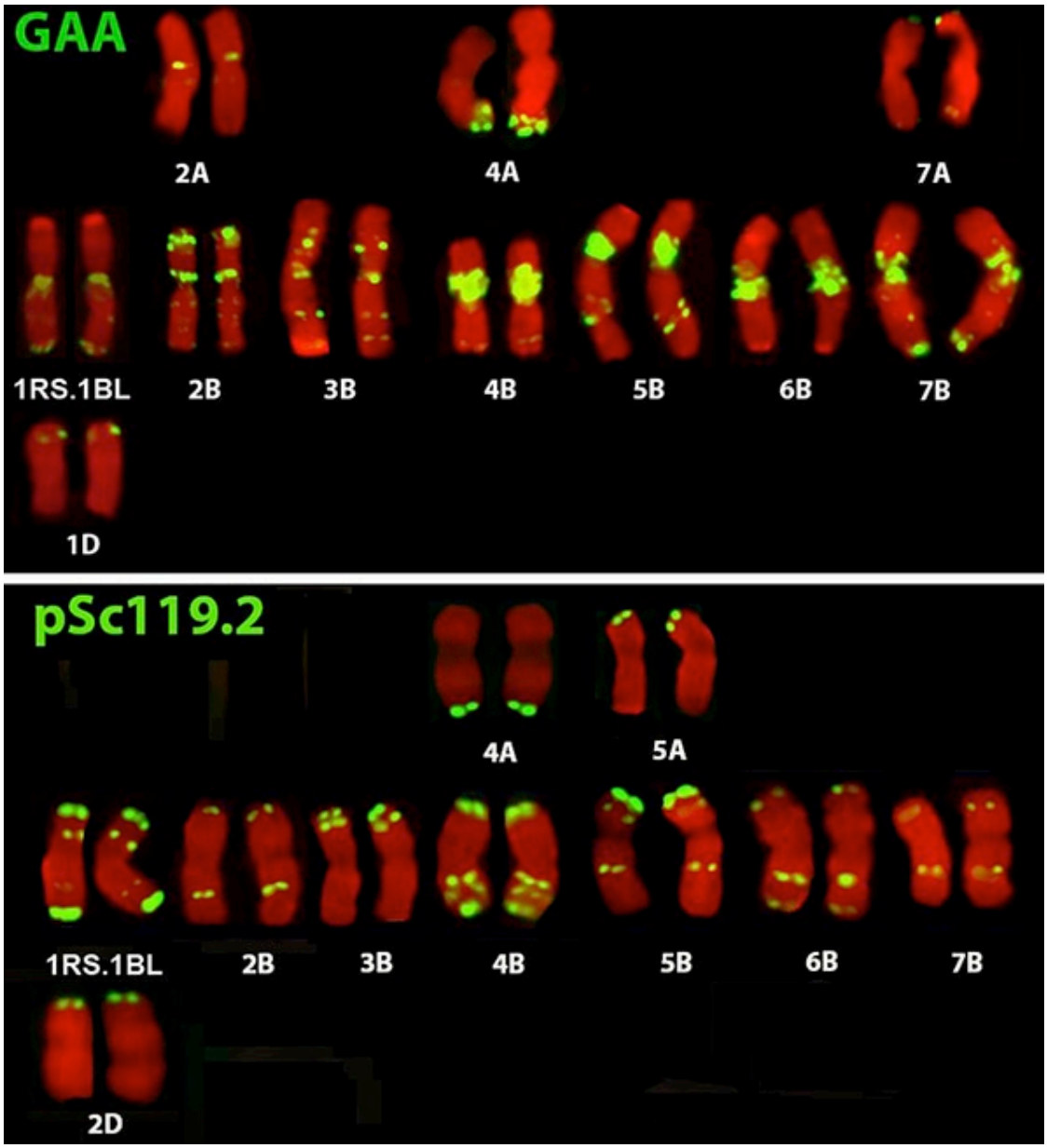


Fig. 3.37. Chromosomes from Prinia wheat showing sites of *in situ* hybridization with GAA (top) and *pSc119.2* (lower panel) in green. The DAPI staining showing chromosome morphology is displayed in red to improve contrast. The 1RS chromosome arm has a characteristic *pSc119.2* hybridization pattern and bright DAPI fluorescence shows the terminal heterochromatin.

Chapter 3: Section II: Karyotype and chromosomal locations of repetitive DNA in the salt tolerant grass *Thinopyrum bessarabicum* (E^bE^b=JJ genome)

3.5 Introduction

Wild grasses belonging to *Thinopyrum* genus (tertiary gene pool) have been acting as potential sources of disease resistance gene for forage and cereal crops (Jiang et al., 1994). Knowledge of their chromosome organizations is crucial for the efficient utilization of this important gene pool in the wheat improvement program (Wang, 1985). Since 1930 breeders have exploited different *Thinopyrum* species after N.V. Tsitsin and his colleagues first showed that these species readily hybridized with various *Triticum* species in the 1930s (Chen, 2005). Breeders have successfully developed several wheat-*Thinopyrum* hybrids to transfer resistance genes into bread wheat. Once a hybrid is produced, it is important to know its karyotype, the parental or ancestral origin of its chromosomes, the stability of its karyotype, and whether there are any translocations or other chromosomal reorganizations (Bie et al., 2007, Kosina and Heslop-Harrison, 1996).

The diploid goat grass *Thinopyrum bessarabicum* (2*n*=2*x*=14, E^bE^b=JJ) is a perennial, rhizomatous maritime wheatgrass distributed around the Black Sea and Mediterranean region. It possesses salinity tolerance and resistance to several diseases, and can be a significant source of genes for wheat improvement (Gorham et al., 1985, King et al., 1997, William and Mujeeb-Kazi, 1993).

In some species of the genus *Thinopyrum*, the localization of rDNA sites and the distribution of highly repetitive DNA sequences have been reported. Dvořák et al. (1989) reported, two 5S rDNA sites in the homoeologous groups 1E and 5E of *E. elongata*. Fominaya et al. (1997) found two 45S rDNA positions were located on chromosomes 5E and of 6E. Lapitan et al. (1987) used the highly repetitive DNA clone *pSc119.2* for the characterization *Thinopyrum* chromosomes and for the identification of the alien chromatin in wheat *Thinopyrum* hybrids.

Brasileiro-Vidal et al. (2003) used 5S and 45S ribosomal DNA sites and the hybridization pattern of the *pSc119.2* and *pAs1* clones to characterize the *T. ponticum*

genome (Brasileiro-Vidal et al., 2003). Very recently the C-banded karyotypes of *Th. bessarabicum* showed diagnostic band-positive sites for each of the seven chromosomes that are quite distinct from those of *T. aestivum* (Mirzaghaderi et al., 2010).

However, a detailed description of the FISH karyotype of the individual chromosomes of *Th. bessarabicum* is still not well defined and published, and this makes it difficult to analyse the progenies of intergeneric hybrids or identify the introgressed alien chromosome segments in a wheat background. Chromosome identification by *in situ* using repetitive DNA clones as a probe is well developed and extensively used in wheat, with multiple karyotypes (Rayburn and Gill, 1987; Pedersen and Langridge, 1997).

3.5.1 Aims

Here we aimed to establish a karyotype showing the major repetitive DNA sequence locations in *Th. bessarabicum*. To detect the possible chromosome polymorphism within the J genome, two accessions of diploid *Th. bessarabicum* with the diverse geographical origin were used.

3.6 Materials and Methods

3.6.1 Plant material

The complete *Th. bessarabicum* disomic and monosomic chromosome addition lines and wheat-*Th. bessarabicum* amphiploid (2*n*=8*x*=56) in a Prinia (bread wheat) background (Zhang et al., 2002) were used for karyotype analysis in the present study. Two accessions of *Th. bessarabicum* with different origin were used for studying chromosome polymorphism: PI 531711 (France), PI 531712 (Tunisia) from the Genebank, USDA-ARS Beltsville, Md., USA.

3.6.2 Methods

Probe labelling, Chromosome preparation and FISH was done using repetitive DNA clones as explained in materials and methods section of Chapter 2.

Table 3.2: *Th. bessarabicum* accessions, Wheat-*Th. bessarabicum* addition lines, amphiploid used in this study

Line	Source	Description/Cross	Chro-moso-me No. (2*n*)
CS	Sears, Missouri	Chinese Spring (bread wheat)	42
Th. bessarabicum	Genebank, (USDA-ARS) PI 531711 (France), PI 531712 (Tunisia)	*Thinopyrum bessarabicum*	14
GH-693 (Group 1J)	CIMMYT	CS/TH.BESS//Gen81/3/*PRINIA	43
GH-615 (Group 2J)	CIMMYT	CS/TH.BESS//Gen81/3/*PRINIA	43
GH-583 (Group 3J)	CIMMYT	CS/TH.BESS//Gen81/3/2*PRINIA/4/ Maize	44
GH-556 (Group 4J)	CIMMYT	CS/TH.BESS//2*Gen81/3/2*PRINIA	44
GH-709 (Group 5J)	CIMMYT	CS/TH.BESS//2*Gen81/3/PRINIA	44
GH-569 (Group 6J)	CIMMYT	CS/TH.BESS//2*Gen81/3/2*PRINIA	43
GH-601 (Group 7J)	CIMMYT	CS/TH.BESS//2*Gen81/3/2*PRINIA/ 4/Maize	44
Wheat-*Th. bessarabicum* amphiploid	CIMMYT		56

3.7 Results

FISH was carried out using repetitive DNA probes to characterize and identify all 14 *Th. bessarabicum* chromosomes. All seven J genome chromosomes carried specific *pSc119.2*, *pTa71* and *pTa794* signals (Fig. 3.38-3.40), making each arm distinguishable from each other and also from the published pattern of the well-known wheat (CS) chromosomes (Mukai et al., 1993, Cuadrado and Jouve, 2008).

The ideogram of the chromosomes of *Th. bessarabicum* was established (Fig. 3.45), in which homologous chromosomes were identified based on their banding pattern and arm ratio. All them J genome chromosomes (1J-7J) arranged and numbered in order of decreasing length irrespective of their homologous relationship with A, B or D chromosomes of wheat (Fig. 3.45). Although the *pTa71* (45S rDNA) hybridization revealed two pairs of satellite chromosomes (Fig. 3.45) but somatic chromosome spread of *Th. bessarabicum* didn't show any chromosome with secondary constriction (Fig. 3.45).

3.7.1 Distribution of pSc119.2, pTa71, pTa794 pattern

According to the present investigations, the *pSc119.2* pattern showed 24 terminal sites (Fig. 3.38, 3.39, 3.45). One-chromosome pair (4J) showed one-intercalary *pSc119.2* bands on the long arm along with *pTa71* site and one strong terminal site on the short arm (Fig. 3.38, 3.39, 3.45), which is similar to the *pSc119.2* pattern of 4B chromosome of wheat (Mukai et al., 1991). One chromosome pair (7J) had a minor terminal *pSc119.2* sites on the short arm while one pair (5J) showed strong terminal *pSc119.2* site. Five pair had telomeric *pSc119.2* site (1J, 2J, 3J, 6J). When studying the distribution of *pSc119.2* in the diploid E genome, Lapitan et al., (1987) found 10 terminal sites plus other sites distributed throughout the entire length of all the chromosomes, except in the telomere of one chromosome. One pair (5J) showed major *pTa71* sites on the short arm similar to wheat chromosome 1B and 6B (Fig. 3.38-3.42, 3.45). The one pair of chromosomes showed *pTa794* minor sites along with *pTa71* sites (Fig. 3.40).

3.7.2 Distribution of dpTa1/Afa/ pAs1 pattern

The tandems arrays of Afa-family repeat are dispersed in several subterminal and interstitial chromosomal regions and have therefore been used as important chromosome markers (Rayburn and Gill, 1986). Here in this study, the *dpTa1/Afa/pAs1* gave signal on all over J genome (Fig. 3.43, 3.44). Similar pattern was found when we use genomic *Th. bessarabicum* genomic DNA as a probe in line 11168 and line 1160 (Fig 3.1, 3.2) The *Th. bessarabicum* DNA strongly labelled D-genome chromosomes. These findings support the fact that *Th. bessarabicum* is very close to D-genome of wheat (see Chapter 3: Section I).

3.8 Discussion

The present study provides a detailed FISH karyotype of the diploid *Th. bessarabicum* JJ genome; previous FISH polymorphism experiments have been performed on other diploid *Th. elongatum* EE genome (Linc et al., 2011). Molecular cytogenetics of an amphiploid between *Triticum durum*, *Thinopyrum distichum*, and *Lophopyrum elongatum* have been studied earlier, and the E genome chromosomes were partially determined by their rDNA loci (Kosina and Heslop-Harrison, 1996).

The C-banded karyotypes of *Th. bessarabicum* showed a distinctive C-banding pattern for each of the seven chromosomes which were quite different from those of *T. aestivum* (Mirzaghaderi et al., 2010). FISH pattern of the *pTa71* probe showed two 45S rDNA sites located in telomeric and subtelomeric regions of the short arms of the E^b chromosomes indicating that *Th. bessarabicum* has two pairs of satellite chromosomes. A similar pattern was observed with C-banding, identifying *Th. bessarabicum* satellite chromosomes (Mirzaghaderi et al., 2010).

We couldn't find any significant chromosome polymorphisms between accession PI 531711 and P1 531712 even though they do not share origin, which is in contrast to Saeidi et al. (2006), Saeidi et al. (2008b) who showed significant differences between subspecies but not within subsp. of D genome using IRAP markers. No physical or genetic map exists for *Th. bessarabicum* so comparison of wheat and *Th. bessarabicum* maps not convenient; translocations with respect to wheat not known. The homology of a number of arms is only known from the addition/substitution/recombinant lines in

wheat but cannot be studied in detail since relevant ditelocentric addition lines are not available.

Two *Th. bessarabicum* accession analyzed here shows a similar pattern with probe *pSc119.2*, which hybridized predominantly to the subtelomeric regions of one or both arms of all chromosomes (Fig. 3.38, 3.39). Additional interstitial signals were observed on the long arm of chromosomes 6 and 7 of the J-genome and chromosomes (Fig. 3.38, 3.39). Thus, these chromosomes can be distinguished using *pSc119.2* alone. This pattern was similar to the pattern Mirzaghaderi et al. (2014) found between two accessions of *Aegilops* (*Ae. umbellulata*, *Ae. triuncialis*). Our result confirms previous observations that probe *dpTa1* is not informative for the identification of J-genome chromosome, as it close to D-genome of wheat and the signals are distributed throughout chromosomes (Liu et al., 2007). No GAA signals were found which was similar to previous findings (Linc et al., 2011, Sepsi et al., 2008). This is probably because centromeric DNA exhibits high diversity and evolution rates, which result in differences in the centromeric DNA even with closely, related species (Heslop-Harrison and Schwarzacher 2011, Heslop-Harrison et al., 2003).

Physical or genetic mapping needs chromosome linkage. Linkage of sequences can be identified by *in situ* by using BAC sequences (BAC-FISH) to known chromosomes by morphology. *In situ* hybridization can also be used to identify evolution and positions of amplification of different sequence classes (e.g. *pSc119.2* terminal on short arms; sequences intercalary in wheat, terminal in rye; microsatellite GAA, ACC locations). The genome of *Th. bessarabicum* has not been sequenced yet. Whole genome sequencing will definitely help to filter and characterize repetitive DNA fraction of the genome, subsequently it will give us more useful *in situ* repetitive sequence. It will not give any translocation breakpoints easily (although identification of translocations in a large-scale genomic sequence is becoming possible in human; Moncunill et al., 2014), and not duplications. The chromosomal banding patterns here will be then useful to characterize addition/translocation chromosomes from *Th. bessarabicum* in wheat and other backgrounds.

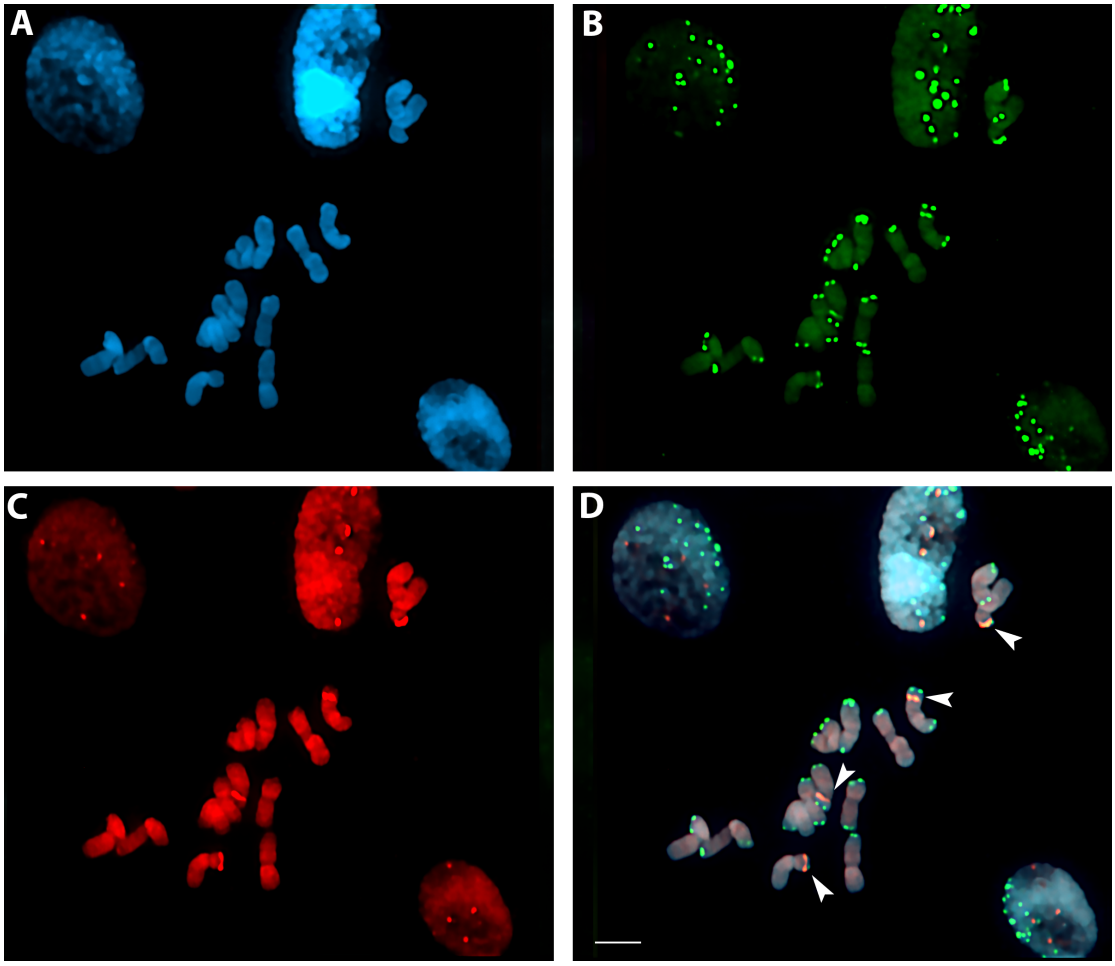


Fig. 3.38: Root tip metaphase chromosomes of the *Th. bessarabicum* accession 531711 (2*n*=2*x*=14, JJ) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence (B) Hybridization pattern of the *pSc119.2* clone labeled with digoxigenin-11-dUTP (detected in green). (C) Hybridization pattern of the *pTa71* clone labeled with biotin-16-dUTP (detected in red). (D) Overlay of A, B and C images. Arrows indicate the 45S rDNA sites.
Scale bar =10μm.

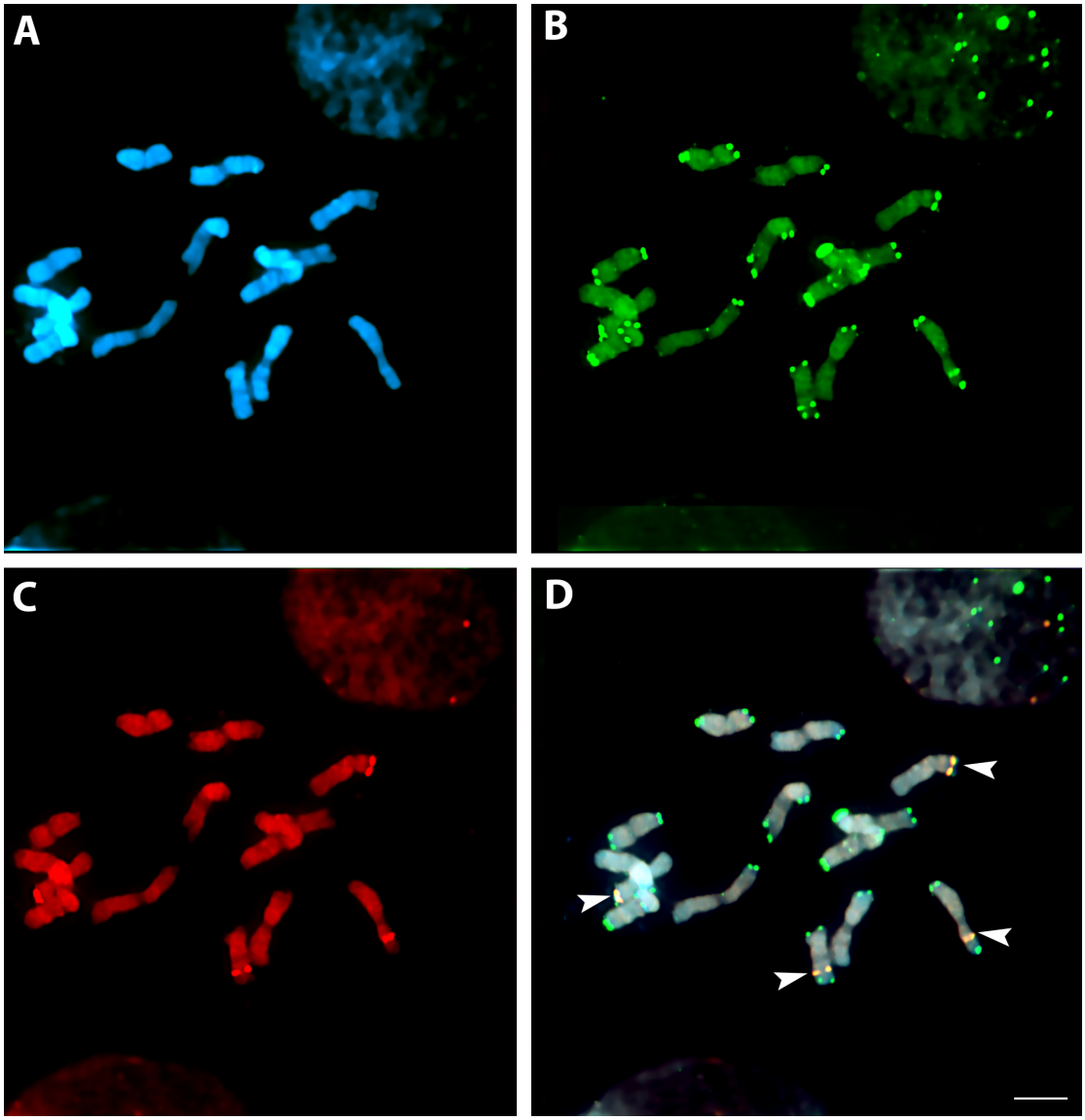


Fig. 3.39: Root tip metaphase chromosomes of the *Th. bessarabicum* accession 531711 (2*n*=2*x*=14, JJ) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence (B) Hybridization pattern of the *pSc119.2* clone labeled with digoxigenin-11-dUTP (detected in green). (C) Hybridization pattern of the *pTa71* clone labeled with biotin-16-dUTP (detected in red). (D) Overlay of A, B and C images. Arrows indicate the 45S rDNA sites.

Scale bar =10μm.

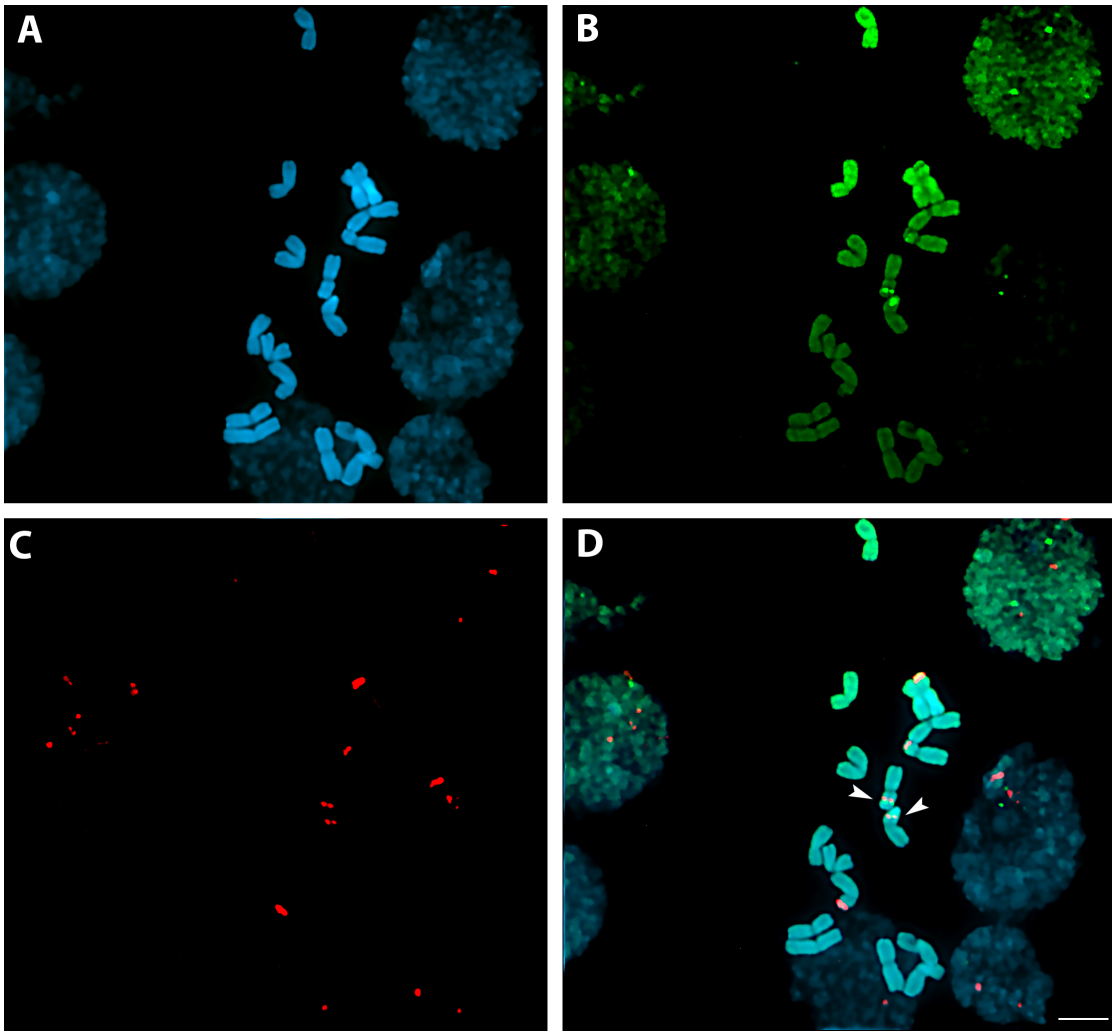


Fig. 3.40: Root tip metaphase chromosomes of the *Th. bessarabicum* accession 531711 (2*n*=2*x*=14, JJ) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence (B) Hybridization pattern of the *pTa794* clone labeled with digoxigenin-11-dUTP (detected in green). (C) Hybridization pattern of the *pTa71* clone labeled with biotin-16-dUTP (detected in red). (D) Overlay of A, B and C images. Arrows indicate the 5S rDNA sites.

Scale bar =10μm.

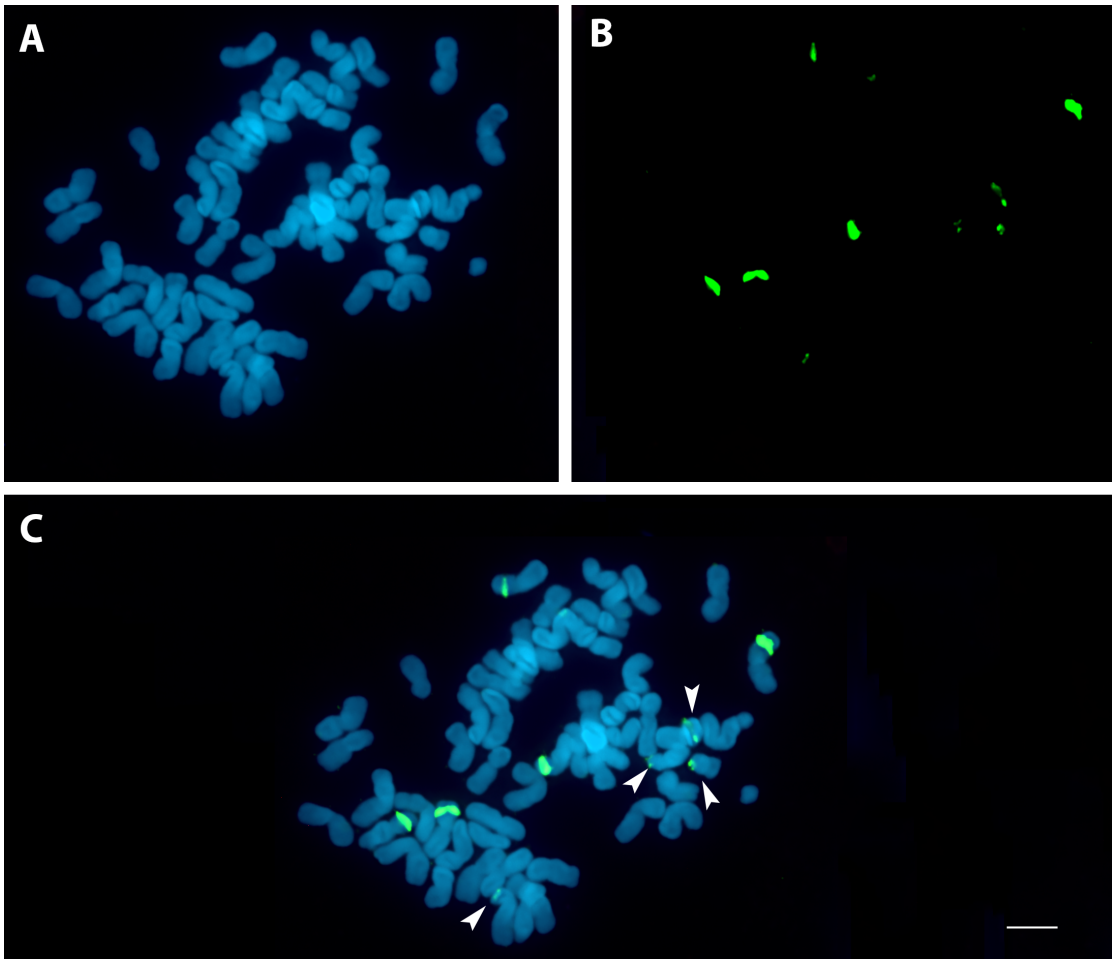


Fig. 3.41: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* amphiploid (2*n*=8*x*=66, AABBDDJJ) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence (B) Hybridization pattern of the *pTa71* clone labeled with digoxigenin-11-dUTP (detected in green). (C) Overlay have A and B images. Arrows indicate the 45S rDNA sites on *Th. bessarabicum* chromosomes. Scale bar =10μm.

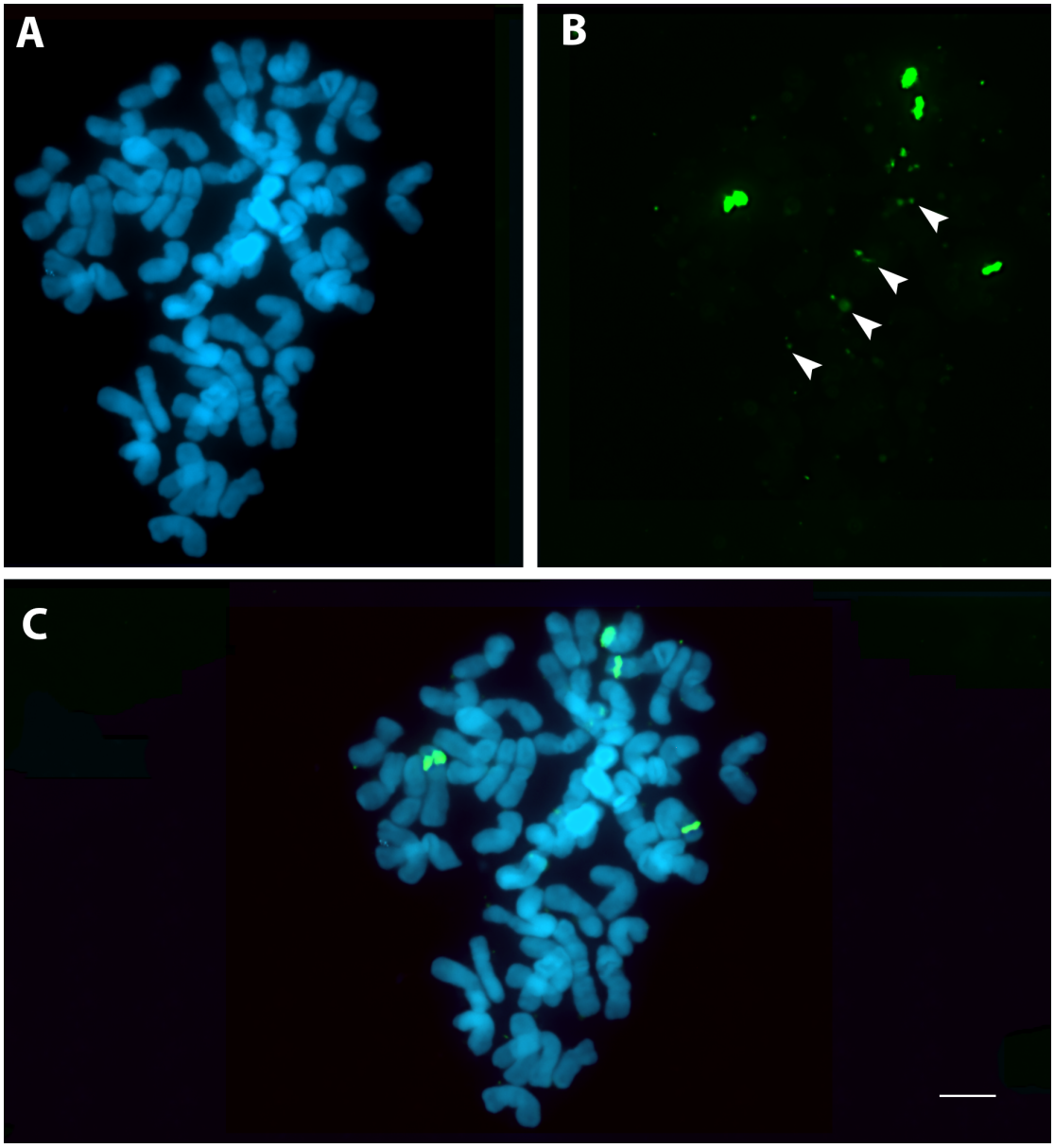


Fig. 3.42: Root tip metaphase chromosomes of the wheat-*Th. bessarabicum* amphiploid (2*n*=8*x*=66, AABBDDJJ) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence (B) Hybridization pattern of the *pTa71* clone labeled with digoxigenin-11-dUTP (detected in green). (C) Overlay have A and B images. Arrows indicate the 45S rDNA sites on *Th. bessarabicum* chromosomes. Scale bar =10μm.

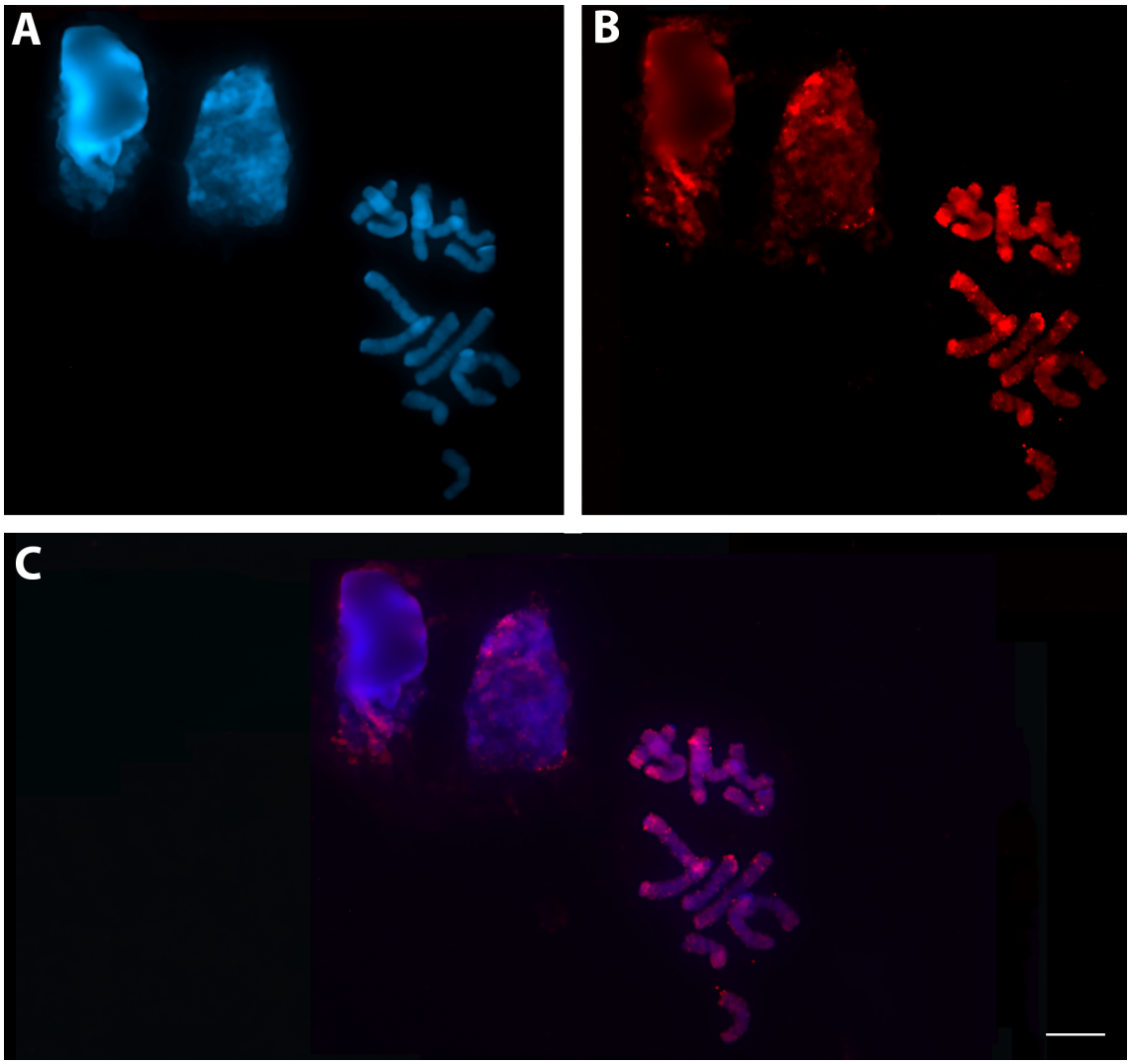


Fig. 3.43: Root tip metaphase chromosomes of the *Th. bessarabicum* accession 531711 (2*n*=2*x*=14, JJ) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence (B) Hybridization pattern of the *dpTa1* clone labeled with biotin-16-dUTP (detected in red). Showing *dpTa1* signals distributed all over chromosomes. (C) Overlay have A and B images. Scale bar =10μm.

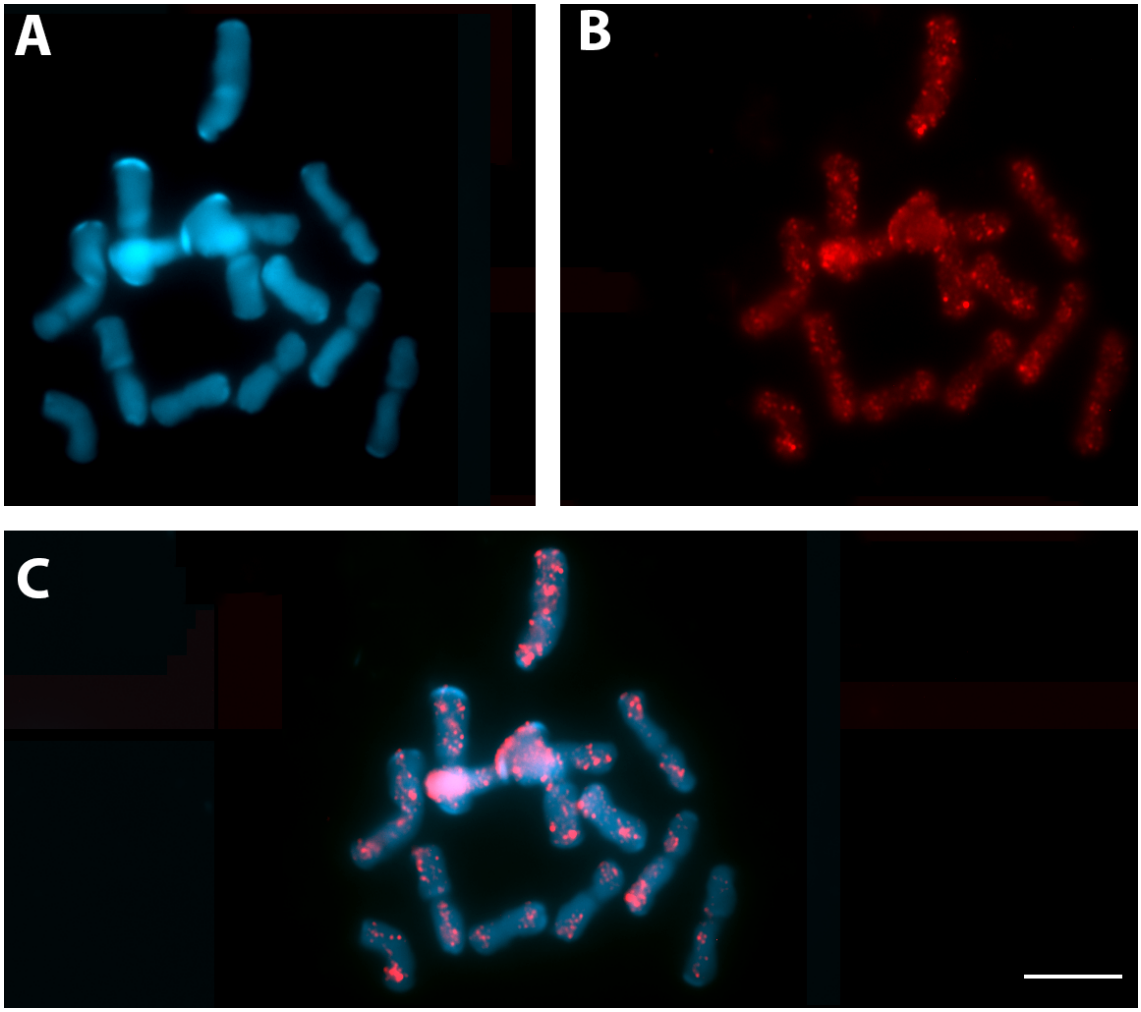


Fig. 3.44: Root tip metaphase chromosomes of the *Th. bessarabicum* accession 531711 (2*n*=2*x*=14, JJ) after fluorescent *in situ* hybridization (FISH). (A) Wheat chromosomes appear in blue with DAPI fluorescence (B) Hybridization pattern of the *dpTa1* clone labeled with biotin-16-dUTP (detected in red). Showing *dpTa1* signals distributed all over chromosomes. (C) Overlay have A and B images. Scale bar =10μm.

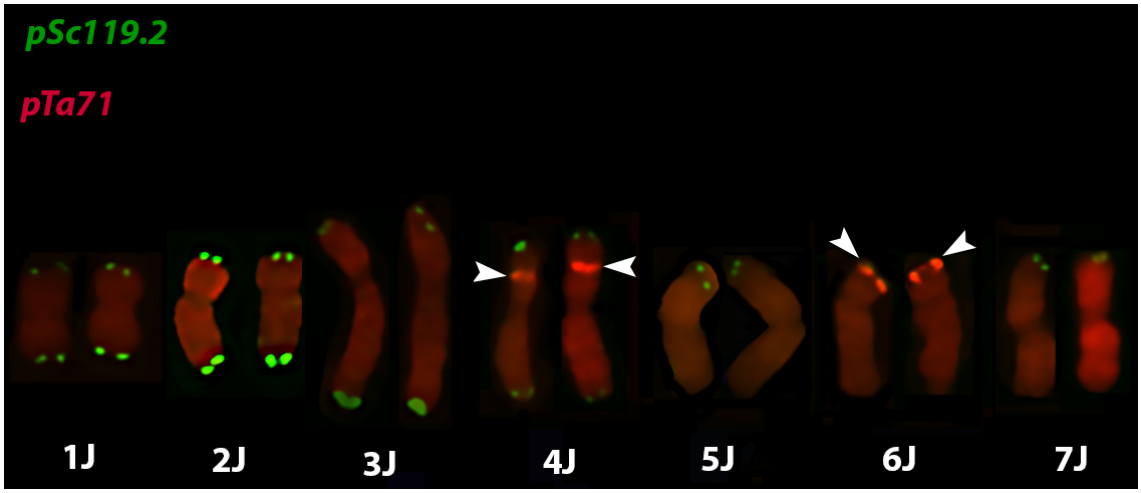


Fig. 3.45: Chromosome karyotype of the *Th. bessarabicum* accession 531711 (2*n*=2*x*=14, JJ) after fluorescent *in situ* hybridization (FISH). Hybridization pattern of the *pSc119.2* clone labeled with biotin-16-dUTP (detected in green). Hybridization pattern of pTa71 9$5S rDNA) probe simultaneously labelled with 50% biotin-16-dUTP and 50% digoxigenin-11-dUTP (Showing in yellow). The DAPI staining showing chromosome morphology is displayed in red to improve contrast. Arrowhead showing *pTa71* sites in yellow.

Chapter 4: Mapping of translocation breakpoints in wheat-*Th. bessarabicum* recombinant lines using SSR markers

4.1 Introduction

In recent years DNA-based molecular markers have been extensively used in bread wheat for the preparation of molecular maps, gene tagging, QTL mapping, DNA fingerprinting, studying population structure and genetic diversity (Song et al., 2005, Gupta et al., 2002, Somers et al., 2004). This has assisted the discovery of new genes controlling phenotypic variation and identification of markers linked to genes for tracking desirable alleles through marker-assisted selection (MAS) in wheat breeding overviewed by Yang et al. (2015). Isolation of informative DNA markers is essential for creating genetic or physical maps. Most widely use DNA markers in cereals are microsatellite or SSR markers (tandem repeats of 2–6 nucleotides), which are consistently found to be more informative than other classes of markers in hexaploid wheat. Their high polymorphism, easy visualization, chromosome specificity, stables and co-dominant nature makes them ideal for molecular mapping analysis (Gupta and Varshney, 2000, Hernández et al., 2002, Kuleung et al., 2004, Gale et al., 1989).

Development of molecular markers in wheat is a relatively complex and tedious process compared to maize, and in particular rice, given the fact that wheat has large genome size, polyploidy, and the high proportion of repetitive DNA (Song et al., 2005). RFLP markers were used at the beginning for the construction of the first molecular genetic map of bread wheat based on the individual homoeologous chromosomes. Followed by PCR-based markers which includes RAPDs, AFLPs and microsatellites (SSRs) and soon became the marker of choice (Chao et al., 1989, Devos and Gale, 1993) (Williams et al., 1990) (Vos et al., 1995) (Röder et al., 1998b).

The construction of molecular maps in wheat started in 1990, with the organization of International Triticeae Mapping Initiative (ITMI), which coordinated the construction of molecular maps of the wheat genome. The ITMI population is a publicly available resource, providing an opportunity to determine the chromosomal location of DNA-based markers with respect to existing wheat molecular markers on a single genetic map (Song et al., 2005). However, due the lack of trait variation the effective application of

the ITMI population and its genetic map in breeding studies is limited (Francki et al., 2009). Numerous laboratories have developed microsatellite markers, which have been explored in various genetic and physical mapping studies (Xgwm: Roder et al. 1998a, 1998b; Xgdm: Pestsova et al. 2000; Xbarc: Song et al. 2005; Xcfa, Xcfd: Sourdille et al. 2004). The first large set of microsatellite markers for the wheat genome has been published in 1998 (Röder et al., 1998b). The molecular genetic map of *Triticum aestivum* contains over 1,500 SSR markers (Bryan et al., 1997, Stephenson et al., 1998, Pestsova et al., 2000, Gupta et al., 2002, Gupta et al., 1999) and USDA at Graingenes 2.0 coordinates the data.

4.1.1 Physical mapping of wheat-alien translocations

Wheat is an well-suited for cytogenetic mapping. It's hexaploidy and large genome size (17 Gb) makes it difficult to find markers closer to the target genes. Aneuploid stocks (nullitetrasomic, di-telosomic, deletion-lines) have been useful in physical mapping of markers specific to chromosome or chromosome arm without the need to identify polymorphism, an especially remarkable achievement in wheat, which exhibits low levels of DNA polymorphism (Endo and Gill, 1996, Sears, 1966). These data has been useful in determining physical positions of genes controlling phenotypic traits and many aspects of wheat chromosome structure and function and evolution (Endo and Gill, 1996). A physical map of 84 deletion lines covering the 21 chromosomes of wheat has been constructed using 725 microsatellites (Sourdille et al., 2004). The BAC library of flow-sorted chromosome 3B of the Chinese Spring assembled into 1036 contig that were anchored with 1443 molecular markers has been used to complete the physical map (Paux et al., 2008).

Wheat-alien translocation has been useful in physical mapping of several disease resistant genes and hence supporting breeding (Mago et al., 2002, Qi et al., 1996). Kynast et al. (2004) used oat-maize addition lines to physically map gene families and markers, which have more than one copy on different chromosomes probably because of the duplicative nature of maize. Castilho et al. (1996) mapped translocation breakpoints in wheat-*Ae. umbellulata* recombinant lines. Crasta et al. (2000) used RFLP markers combined with GISH to analyze the progeny of γ -irradiated wheat – *Th. intermedium* introgression lines to identify and characterize *Th. intermedium* chromosome segments. Similarly, Dundas et al. (2015) physically mapped *Th. ponticum* translocation 6Ae#1L in wheat carrying rust resistance gene *Sr26*. Shen et al. (2013)

mapped blue-grained gene derived from *Th. bessarabicum* using SSR markers. Ye et al. (2015) mapped translocation breakpoints in wheat-*Ag. cristatum* 5A/6P translocation lines using SSR markers.

4.1.2 Transferability of wheat anonymous SSR and EST-SSR markers

Most SSR markers are genome-specific and their transferability across related species is low (Mullan et al., 2005). Nearly 50% of the wheat SSR markers are genome-specific meaning they detect only a specific locus on one of the three genomes, when isolated from a SSR-enriched genomic library (Chao et al., 1989, Bryan et al., 1997). We generally expect amplification of a single band or co-migrating twin bands with a single gSSR primer pair, if they amplify from more than one of the three wheat genomes; the amplified fragments are often clearly distinguishable on high-resolution gels or sequence fragment-length measuring platforms.

SSR markers are more advantageous than the bi-allelic markers due to the fact that they can detect up to more than 30 different alleles in the wheat germplasm for a given locus and hence are multi-allelic (Plaschke et al., 1995, Röder et al., 2002). The high polymorphism of SSRs makes them less suitable for comparative studies outside the species, for example with wild relatives (Saeidi et al., 2006, Saeidi et al., 2008a). The disadvantage of using microsatellite markers in wheat chromosomes is that these cannot be distinguished from the genes present at the physical end of the chromosomes. However, the SSR markers used currently are not localized in the genes. Thus generating interest in developing EST-SSRs specifically targeting SSR polymorphisms related to genes (Röder et al., 1998a).

On the existing microsatellite map of wheat, the B genome has the highest and the D genome has the lowest number of microsatellite loci (Pestsova et al., 2000). In wheat breeding, SSRs are being used as the marker backbone for a variety of purposes although other genotyping platforms including arrays such as Axiom arrays and sequencing approaches (see Chapter 6) are increasingly being exploited. These include the localization of individual genes and large set of QTLs (quantitative trait loci) onto the 21 wheat chromosomes affecting morphological and agronomically important traits (Perretant et al., 2000, Huang et al., 2003, Huang et al., 2004, Börner et al., 2002).

Conventional experimental methods for developing SSRs required enriched libraries or the screening of genomic libraries including sequencing clones containing putative SSR

tracts, together with designing and testing flanking primers. However, the time and costs necessary to identify the sequence containing SSRs, high redundancy frequency among the clones and the location of the microsatellite and primer designing of the flanking sequences have prevented the broad use of microsatellites in plants (Beckmann and Soller, 1990, Röder et al., 1995). To overcome this problem, various pre and post-cloning procedures (Powell et al., 1996) to create genomic libraries enriched for SSRs have been developed. Developing EST sequences databases is feasible option to solve this problem (Nicot et al., 2004).

The availability of cereal EST sequence database in the public domain has provided a valuable resource of non-anonymous DNA-markers (EST-SSRs). ESTs are being sequenced from these species. An international collaboration, the International Triticeae EST Cooperative (ITEC, growing from some initiatives from the early 1990s) was established in 1999 to develop a large EST database from wheat and barley (http://wheat.pw.usda.gov.genome/). Although EST-SSRs were shown to be less polymorphic markers than genomic SSR (gSSR) markers (Becker and Heun, 1995, Eujayl et al., 2002) which makes them difficult to map, they have other advantages over those from traditional enriched genomic libraries. The great potential of these markers is due to their physical association with coding regions of the genome, which may correspond directly to genes controlling agronomically important traits of interest provides a map location of genes (Holton et al., 2002, Gadaleta et al., 2009), or at least tends to place the markers in gene-rich genomic regions. These markers have greater transferability between species, which may be possible because of higher conservation of SSR-flanking sequences within ESTs.

4.1.3 EST-SSR markers in Thinopyrum

Several DNA markers, for example randomly amplified polymorphic DNA (RAPD), sequence characterized amplified region (SCAR), microsatellite markers or simple sequence repeat (SSR) were widely applied to develop genome- or chromosome-specific markers for *Th. elongatum* species (Chen et al., 2013, Ming-Shan et al., 2004, Zhang et al., 2008, Xu et al., 2012). The development EST-based PCR markers is a more recently developed approach to identifying markers for detecting alien chromatin in wheat, their transferability among the related species allowed it to track introgressed alien chromatin easily (Varshney et al., 2005, Scoles et al., 2009, Mullan et al., 2005). For example, using 41 SSR developed from wheat ESTs Mullan et al. (2005)

successfully detect *L. elongatum* loci in wheat. The WYMV-resistant terminal translocation line NAU421 with the shortest introduced 4VS (*Haynaldia villosa*) fragment was detected using five EST-derived SSR markers (Zhao et al., 2013). A high degree of DNA sequence diversity and synteny exists between wheat and many of its relatives (Mullan et al., 2005, Appels et al., 2003, Devos and Gale, 1997, Dvořák, 1980, Gustafson et al., 2010). Molecular markers based on the synteny of different genomes are useful for determining the homoeologous relationships of chromosomes from different species by comparative mapping (Van Deynze et al., 1998, Heslop-Harrison, 2000).

The conserved orthologous set (COS) markers, designed based on rice syntenic regions and presumably amplifying fragments corresponding to the similar linkage group(s) of related plants, had been utilized for comparative genomics in higher plants (Fulton et al., 2002; Tomaszewski et al. 2012) and mapping quantitative trait loci in wheat (Quraishi et al., 2009). The synteny between rice and wheat homologous groups was evident between some rice and *Thinopyrum* homologous groups. *Oryza* homologous groups 4, 7, 8, 9, and 11 showed strong relationships with *Thinopyrum* homologous groups 2, 2, 7, 5, and 4, respectively. A few primer pairs corresponding to rice group 10 chromosome were used to assess its homoeologous relationship with *Thinopyrum* chromosomes (Gustafson et al., 2010). Francki et al. (1997) have used this information to characterize *Th. intermedium* alien chromosome in P29, a wheat disomic–alien substitution line. Guo et al. (2015) compared chromosomes 7el1, 7el2, 7Ee, and 7Ei derived from *Thinopyrum* using COS (Conserved orthologous set) markers and GISH. Thus, there have been a number of developments in the past 10 years leading to the development of transferrable markers in wheat, contrasting with earlier COS and SSR probes which were more restricted to single genomes.

4.1.4 Potential of SSR markers in mapping alien genes

The PCR-based molecular markers are useful in marker-assisted selection (MAS) in breeding program these days. Markers help in the identification and tracking of the alien genes introgressed from the wild relatives associated with beneficial traits. Since conventional selection based on plant phenotypes is bulky and inefficient, markers can be used for the screening of desirable recombinant plants with the markers of interest. Considering the usefulness of markers it becomes essential to develop wheat-alien map using new set of PCR-based molecular markers specific to the alien chromosome to

detect the specific translocations and possible recombinants to accelerate selection in a breeding program (Qi et al., 2008). Translocation breakpoints of interspecific chromosome rearrangements can be determined exactly using cytogenetic methods, e.g. genomic *in situ* hybridization (GISH) (Le et al. 1989; Schwarzacher et al. 1989; 1992).

Tracking of such introgressions in offspring families may also provide insight into homeologous recombination leading to incorporation of the desired genes from the alien donor into the recipient crop while eventually losing genes controlling unwanted traits.

4.1.5 Aims

Here the aim of this study was to test markers on wheat-*Th. bessarabicum* introgression lines and then exploit the amplification and polymorphisms to identify alien chromosome segments and map wheat-alien chromosomal translocation breakpoints. The cytogenetic data (Chapter 3) allowed the choice of markers to be closely targeted to the genomic regions involved in the translocation.

4.2 Materials and methods

4.2.1 DNA extraction and PCR amplification

DNA was isolated from the wheat-*Th. bessarabicum* translocation lines, *Thinopyrum bessarabicum* (NPGS accession No. PI 531711), Chinese spring and Chinese spring nullisomic-tetrasomic lines as mentioned in Chapter 2 (Materials and methods). 75 wheat microsatellite primers were selected from the GrainGenes 2.0 database (http://wheat.pw.usda.gov/GG2/index.shtml; gwm: Röder et al. 1998a, 1998b; barc, wmc: Somers et al. 2004; gdm: Pestsova et al. 2000; cfa, cfd: Sourdille et al. 2004). The amplification was done by PCR using Tprofessional Gradient Thermocycler (Biometra) in a 15 µl reaction mixture containing 50 ng of template DNA. Kapa Taq was used, Kapa 1x Kapa biosystems bufferA (750 mM Tris-HCL pH 8.8, 200mM $(NH_4)_2SO_4$, 15mM $MgCl_2$, 200 µM of dNTPs (Bioline), 0.6 µM of each primer and 0.5 U of Kapa Taq DNA polymerase was used. PCR conditions were kept similar for all markers except number of cycles which were; 94^{o}C for 4 min, followed by 35-40 cycles at 94^{o}C for 1 min, 52-63^{o}C (depending upon annealing temperature (Tm) of different primer sets (see Appendix 1) for 45 secs, 72^{o}C for 2 mins, and final extension of 72^{o}C for 10 mins followed by holding block at 16^{o}C. Amplification and polymorphism of the PCR

products were analyzed by 1.5-3% agarose gel as given in Chapter 2. Gel images were analyzed and processed using Adobe Photoshop CC.

4.3 Results

4.3.1 SSR marker analysis of line 1160

Fourteen markers specific to the wheat chromosome arm 4BL were used in the present study in order to identify translocated chromosome in translocation line1160. Two (Xgwm375 and Xwmc546) out of the fourteen markers tested showed no polymorphism between wheat Chinese Spring and the diploid genome of *Th. bessarabicum* (Fig. 4.1, 4.2) so these markers could not be used to characterize the translocation (Table 3). Eleven markers amplified polymorphic loci from both the wheat as well as *Th. bessarabicum* chromosomes. Two markers (Xgwm149 and Xgwm538) showed polymorphism only in *Th. bessarabicum* not in wheat (Fig. 4.1). Two markers (Xwmc710, Xgwm611) were polymorphic only in wheat and not in *Th. bessarabicum*. All the polymorphic markers amplified in both Chinese spring as well as translocation line 1160 so couldn't able to define translocations. Two markers (Xgpw1144, Xgpw1113) didn't amplify in translocation line 1160 (Fig. 4.2).

4.3.2 SSR marker analysis of line 1164

Eleven microsatellites mapped to the wheat chromosome 6BL were used to confirm the presence of the translocation 6BS.6BL-6JL. These markers were only able to detect missing wheat chromosome but couldn't detect the presence of alien chromosome segment. Seven of them (Xwmc539, Xgwm147, Xbar178, Xbarc134, Xwnc726, Xgwm219, Xgwm626) (Fig. 4.3, 4.4) were polymorphic amplified more than one band in wheat and translocation line (1172). Marker Xwmc152 didn't show any polymorphism so couldn't used to describe the translocation. The translocation line 1164 lacked the 6BL-specific fragments produced by marker Xgwm626 on wheat DNA (187bp, Fig. 4.3).

4.3.3 SSR marker analysis of line 1168

Eight SSR markers mapped to the wheat chromosome 5AL were used to confirm the presence of the T5AS.5AL-5JL translocation. Here, the deletion of a part of a wheat chromosome can be detected with the SSR markers and not the presence of alien segment. It would be possible if we had *Thinopyrum* specific markers. Seven markers

were polymorphic and amplified more than one band in wheat and translocation line 1168 so couldn't able to detect translocation.

4.3.4 SSR marker analysis of line 1172

Sixteen SSR markers mapped to the terminal region of wheat chromosome 5DS were used to assign 5DL.5DS-5JS translocation breakpoints. All of them were polymorphic so were very suitable for describing the translocation. The translocation line 1172 lacked the 5DS-specific fragments produced by Xgwm190, Xcfd189, Xcfd67, Xgpw326 and Xwmc233 on wheat DNA (201bp, 280bp, 180bp, 147bp and 260bp respectively (Fig. 4.5, 4.6 and 4.7). Xwmc233 is known to have 2 loci within the wheat. The markers mapped to the deletion bin 5DS2-0.78-1.00 and 5DS5-0.67-0.78 (http://wheat.pw.usda.gov/cgi-bin/cmap) could not amplify in translocation line 1172 but amplified in wheat confirming the presence of translocation. Markers mapped to deletion bin C-5DS1-0.63 were amplified both in wheat and in translocation line 1172 (Fig. 4.9).

4.3.5 SSR marker analysis of line 1176

Analysis of translocation line 1176 was very critical, even with FISH and GISH. Based on initial FISH screening, we thought that chromosome involved in translocation is either 3BL or 7BL, so we applied 7BL and 3BL and specific SSR markers, But as not expected they amplified both in translocation line (line 1176) and in Chinese Spring. After more detailed FISH analysis using more repetitive probes, we concluded that chromosome involved in translocation is 2BL. Seven 2BL specific markers were applied to confirm the presence of the T2BS.2BL-2JL in line 1176, these markers could only detect missing part of wheat chromosome and not the presence of alien chromosome. Only three markers were polymorphic but amplified in both parent and in translocation line, these markers were suitable to detect translocations.

4.3.6 SSR marker analysis of line 1180

Analysis of translocation line 1180 was very straightforward, as initial FISH screening showed that this is a whole arm translocation. Seven SSR makers mapped to the wheat chromosome arm 1AS were used to confirm the 1JS.1AL translocation. Four out of seven makers (Xgwm136, Xgwm36, Xwmc24, Xbarc28) were polymorphic and failed

to amplify in translocation line 1180, but they were amplified in both wheat and background Prinia (Fig. 4.8).

4.4 Discussion

The present study demonstrates that *in situ* hybridization techniques (Chapter 3), combined with SSR marker analysis, are extremely useful in detecting and identifying specific alien chromosome involved in translocation, leading to the selection of genetic materials useful for future mapping studies. We have successfully mapped T5DL.5DS-5JS in line 1172 using 16 SSR polymorphic markers (Fig. 4.9). The marker order is similar to the SSR based deletion bin map developed by Sourdille et al. (2004), with one exception, that Xcfd189 and Xgwm190 mapped distal to Xcfd18 on the map by (Sourdille et al., 2004), whereas Xcfd189 and Xgwm190 were located proximal to Xcfd18 in our map (Fig. 4.9). Two markers (Xgpw1144, Xgpw1113) couldn't amplify in translocation line 1160 (Fig. 4.2) and these markers were initially mapped to terminally on the long arm of wheat chromosome 4BL (Sourdille et al., 2004), proving that terminal portion of wheat 4BL is missing and it's replaced by *Th. bessarabicum*. The 6BL-specific fragments produced by marker Xgwm626 was absent in the translocation line 1164 (Fig. 4.3). Marker Xgwm626 was initially mapped terminally at chromosome 6BL (Sourdille et al., 2004) hence proved that *Th. bessarabicum* chromatin was present on the long arm of wheat chromosome 6BL.

We couldn't map breakpoints in translocation line 1160, 1164, 1168 and 1176 because the use of molecular markers is very limited when small fragments of related species are achieved in bread wheat (Fig. 4.1, 4.2, 4.3, 4.4, 4.5). Most of the markers weren't polymorphic between wheat and *Th. bessarabicum* hence weren't suitable to map translocation breakpoints in these lines. Ali (2012) successfully used 26-markers polymorphic between wheat and *Th. intermedium* to map recombinant 1BS chromosome. In line 1176 we found markers polymorphic between Chinese spring and *Th. bessarabicum* and was potentially useful in mapping translocation T5DL.5DS-5JS (Fig. 4.9).

Genetic maps of wild-relative species are not saturated either, so it is often difficult to screen alien genetic introgressions from relative species based only on molecular markers. Furthermore, the use of molecular markers is based on the previous knowledge of the exact chromosome introgression but can be useless when the chromosome or

chromosome segment from the relative species involved in recombination are not well characterized or chromosome introgressions from the relative species occurred randomly in the wheat background. The wheat parents (or close relatives) used for generating introgression lines may not be included in the original panel for development of SSR markers, so new alleles, or no amplification, may be found. In this study, we faced difficulty in mapping translocation breakpoints, as there is a very little knowledge about molecular markers in *Th. bessarabicum*. Qi et al. (2007) had serious problems to detect recombinants because there were not enough molecular markers to determine the presence of *Th. intermedium* in wheat. They used more than 16,000 EST loci to define the *Th. intermedium* specific chromosome regions in wheat. Unfortunately, only nine STS markers were polymorphic between the *Th. intermedium* and wheat.

The physical and genetic maps show same markers order along the chromosome, when comparing genetic and physical maps, discrepancies were found in marker order and in the distances between markers. The physical location of most markers is unknown except for specific points such as the centromeres and secondary constrictions (Lukaszewski and Curtis, 1993, Heslop-Harrison, 1991). When physical and genetical locations are known, there is often little correlation between the separation distances of markers on the two types of map. With existing recombination hotspots, which are often in the gene-rich regions close to the telomeres, genetic maps only provide an estimate of marker positions (Gustafson et al., 1990, Lukaszewski and Curtis 1993). It is particularly difficult to assess the marker order using genetic mapping in regions of high marker density, where genetic distances are very short (Sourdille et al., 2004).

The size of an alien introgression can be highly variable which corresponds to the compatible region for recombination, considering alien chromosomes are rearranged relative to homologous chromosomes of wheat. The discrepancies between genetic and physical maps can be attributed to the uneven distribution of wheat-alien recombination along chromosome length, mostly at telomeric regions (Devos et al., 1993, Lukaszewski and Curtis, 1993).

Other factors like genotyping error, undetected chromosome rearrangements (such as translocation, deletion and inversions), low map coverage, duplicated marker loci and segregation distortion may cause inconsistent marker order in genetic maps and physical maps (Francki et al., 2009, Shearman et al., 2015, Staňková et al., 2015). Tsujimoto and Noda (1990) have studied the lines of wheat having short deletions,

detected by C-banding on the long arm of the chromosome-designated 5A. They found that a deletion of 13% of the physical length of the arm caused loss of markers representing at least 83% of the genetic length of the arm. It is notable that the challenge of miss-match between genetic and physical lengths was first pursued 25 years ago, and only now is being recognized as a constraint on exploiting DNA-based markers to understand and characterize alien-recombinant introgression lines.

The molecular markers (SSR, RAPD, RFLP, AFLP) have been extensively used for detection of introgressed chromosome segments. In a translocation line, if the recombination between the alien chromosome introduced to the bread wheat genome is absent and this translocation is stably inherited, a single MAS or MAB marker is sufficient to identify the alien chromosome (Timonova et al., 2013). However, their use to detect and define introgressions and chromosome rearrangements is limited due to low marker placement accuracy and even lack of specific markers (Víquez-Zamora et al., 2013, Anderson et al., 2011, Kumar et al., 2012, Aflitos et al., 2015). In addition, sequence duplications, heterozygosity, and discrepancies between genetic and physical maps can seriously hamper data interpretation (see discussion). Such limitations can partly be overcome through the use of cytogenetic techniques such as genomic *in situ* hybridization (GISH) and fluorescent *in situ* hybridization (FISH). GISH can be used to obtain information on the size and number of alien chromosomes or chromosome segments, interspecific and intergeneric translocations resulting from homeologous recombination, and the presence and approximate location of introgressed genes (Schwarzacher et al., 1992). By using markers only it is difficult to determine the marker location on the chromosome. Therefore, combining marker and cytological methods (FISH, GISH) will increase the possibility of detecting chromosome rearrangements as well as assist in determining chromosome identity and marker order (Pu et al., 2015).

Here, *in situ* hybridization enables the determination of the exact chromosomal composition of the chromosome arms involved in wheat-*Th. bessarabicum* translocation lines (see Chapter 3). Such strategies, however, are not sufficient for unraveling complex rearrangements and identification of chromosome breakpoints at nucleotide accuracy. Identification of alien genomic introgressions can be difficult, especially when the genomic introgressions have occurred randomly. The high level of synteny among related species and the complexity of the wheat genome also restrict the use of

molecular markers in detecting alien introgression (Salse and Feuillet, 2007). The use of molecular markers combined with *in situ* hybridization is very useful to detect and define alien chromosome introgressions, but the alien chromosome fragment needs to be well characterized in order to select specific molecular marker to be unambiguously distinguished from the same chromosome region in related species (Schwarzacher et al., 1989, Calderón et al., 2012, Zhao et al., 2013). Here, we have identified and tested several potential molecular markers which are polymorphic between Chinese spring and *Th. bessarabicum* (Fig. 4.9) which will be useful in future breeding and wheat improvement programs for fast and verifiable identification of small translocations or introgressions.

Other methods for introgression detection include restriction site associated DNA (RAD) and genotyping by sequencing (GBS) (see Chapter 6) will be more accurate and helpful in defining and characterizing alien introgressions. Whole genomes sequencing with mid-length reads and limited assembly is likely to find the regions homoeologous to existing markers from other genomes and hence, even without a GBS approach, allow markers to be exploited in alien species by their homology to mapped wheat markers. The applications and approaches are still being developed, the costs are expensive (although once developed can be automated rather than requiring skill), and as yet the interpretation is not yet routine. The use of the targeted markers based on cytogenetic analysis here shows accurately the breakpoint locations, can be integrated with genetic maps (molecular or field performance), and then knowledge can be applied to screen higher numbers of lines with low-cost DNA markers such as the SSRs.

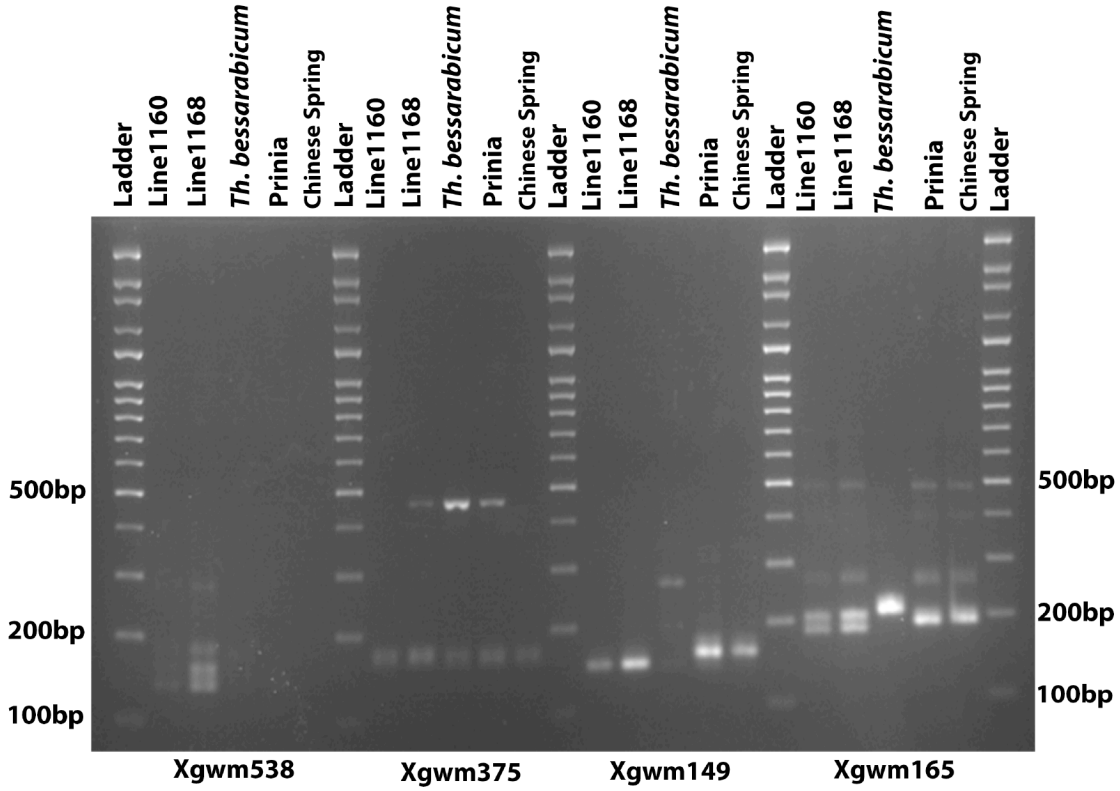


Fig. 4.1: Gel image showing PCR amplification of the wheat-*Th. bessarabicum* introgression line 1160 (T4BS.4BL-4JL) using SSR markers (A) Xgwm538 present in line1160 and missing in parents (b) Xgwm375 missing in line 1160 and present in parents (C) Xgwm149 missing in line 1160 and parents in parents. (D) Xgwm165 present in both parents as well as translocation line 1160.

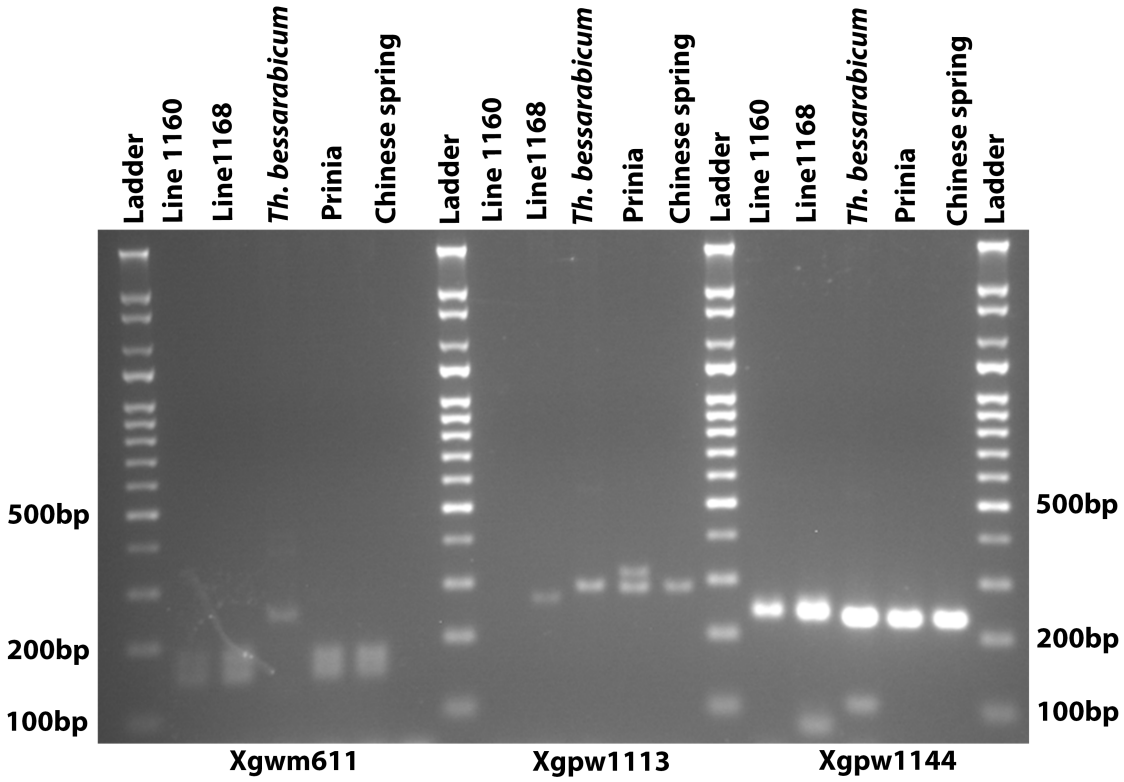


Fig. 4.2: Gel image showing PCR amplification of the wheat-*Th. bessarabicum* introgression line 1160 (T4BS.4BL-4JL) using SSR markers (A) Xgwm611 present in CS and line 1160 (B) Xgpw1113 absent in line1160 but and present in parent CS (C) Xgpw1114 present in line 1160 but absent parents.

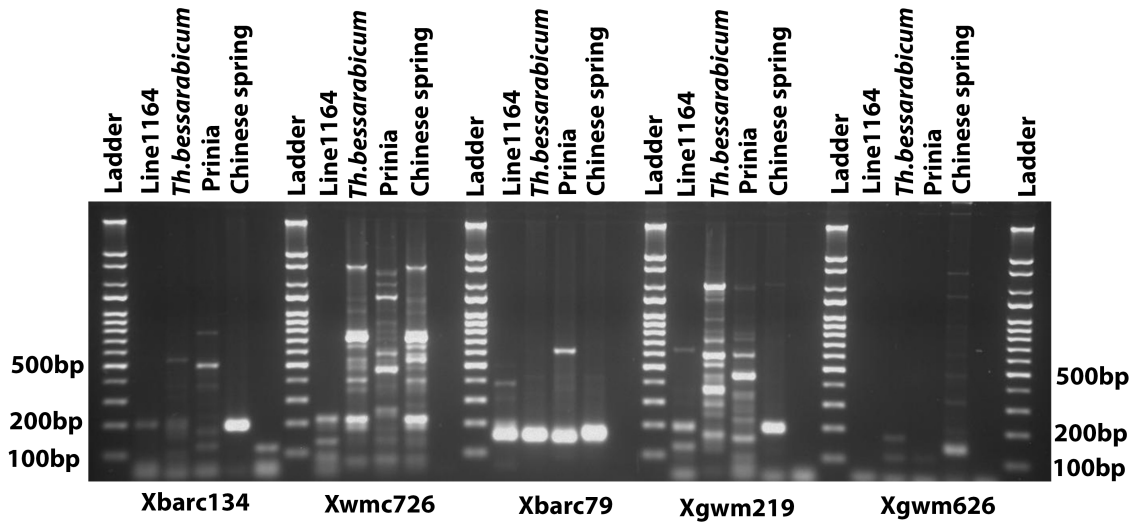


Fig. 4.3: Gel image showing PCR amplification of the wheat-*Th. bessarabicum* introgression line 1164 (T6BS.6BL-6JL) using SSR markers (A) Xbarc134 present in CS and line1164 (b) Xwmc726 present in line 1164 and parents (C) Xbarc179 present in line 1164 and parents (D) Xgwm219 missing in line 1164 but present in parents (E) Xgwm626 missing in translocation line 1164 but present in parents (CS and *Th. bessarabicum*).

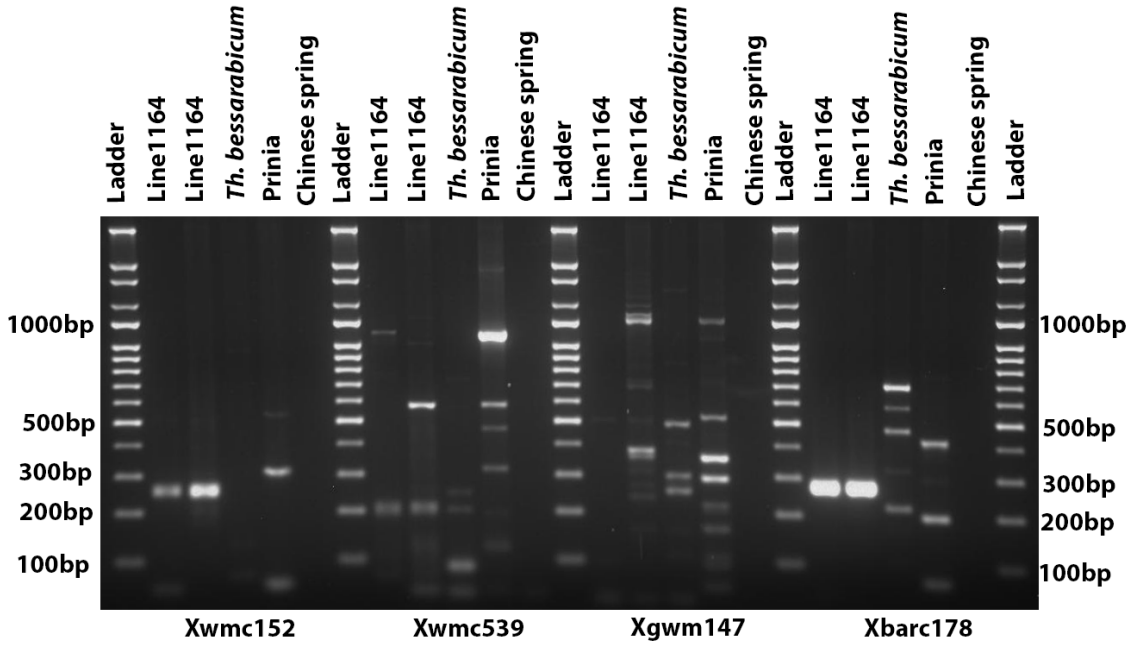


Fig. 4.4: Gel image showing PCR amplification of the wheat-*Th. bessarabicum* introgression line 1164 (T6BS.6BL-6JL) using SSR markers (A) Xwmc152 is present in CS and line1164 (b) Xwmc539 is present in line 1164 and parents (C) Xgwm147 present in line1164 and parents (D) Xbarc178 is present in line1164 and parents.

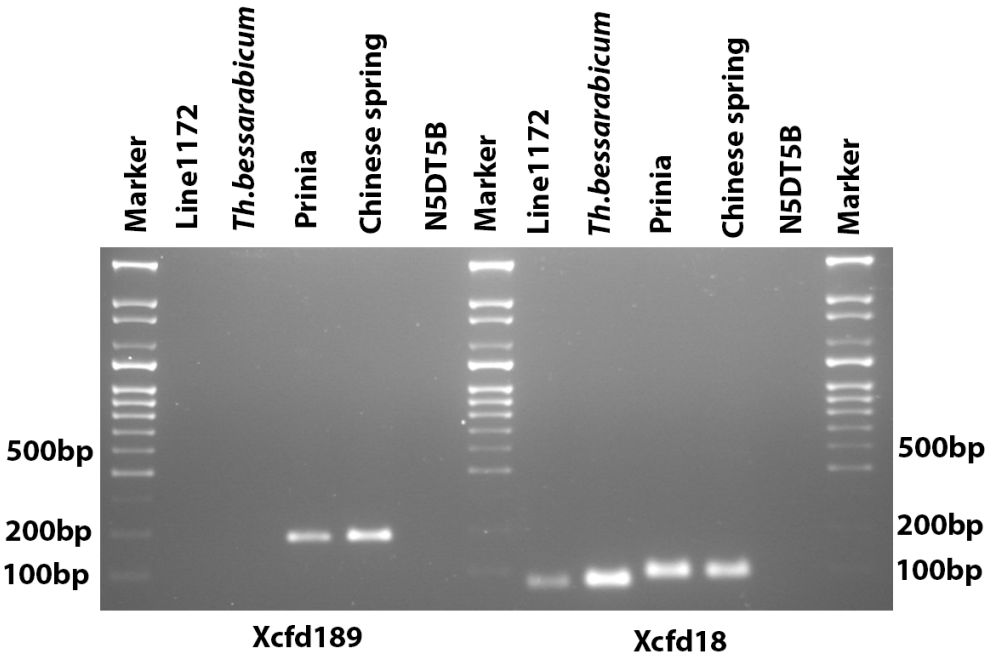


Fig. 4.5: Gel image showing PCR amplification of the wheat-*Th. bessarabicum* introgression line 1172 (T5DL.5DS-5JS) using SSR markers (a) Xcfd189 present in CS and missing in translocation line 1172 and in parents (*Th. bessarabicum* and Prinia) (b) Xcfd18 present in CS and Prinia missing in translocation line 1172 and *Th. bessarabicum*.

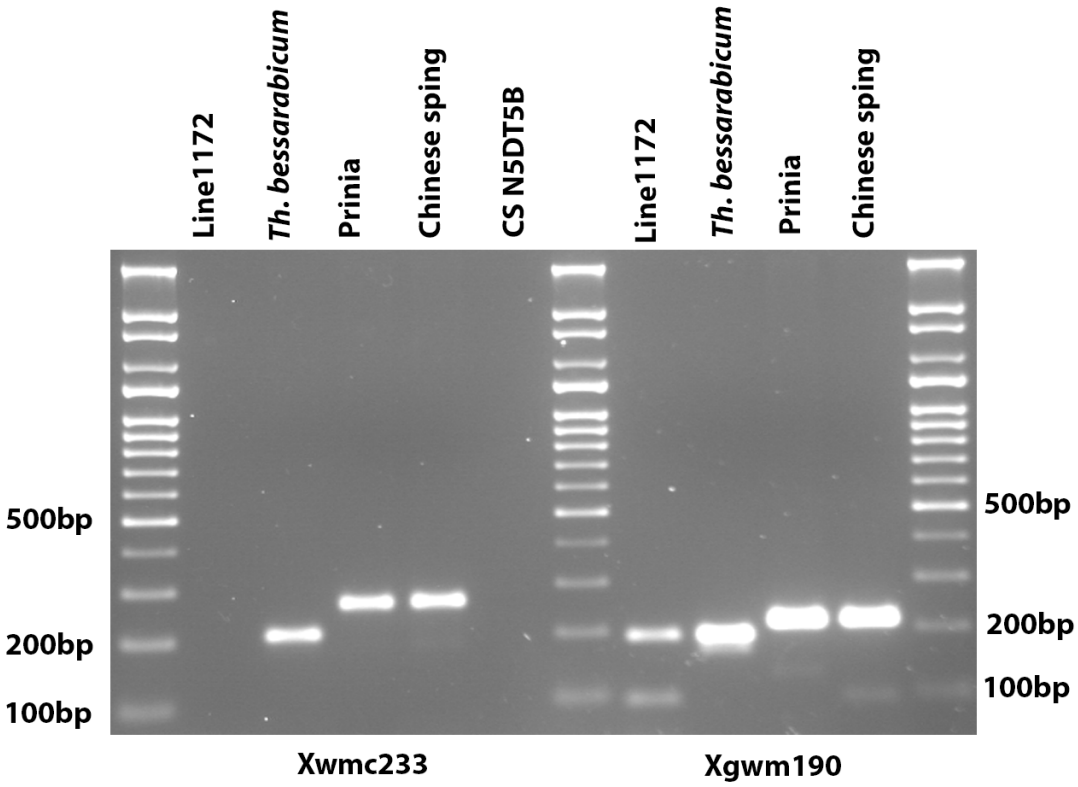


Fig. 4.6: Gel image showing PCR amplification of the wheat-*Th. bessarabicum* introgression line 1172 (T5DL.5DS-5JS) using SSR markers (a) Xwmc233 present in CS and Prinia but missing in translocation line 1172 (b) Xgwm190 present in CS and Prinia missing in translocation line 1172 and *Th. bessarabicum*.

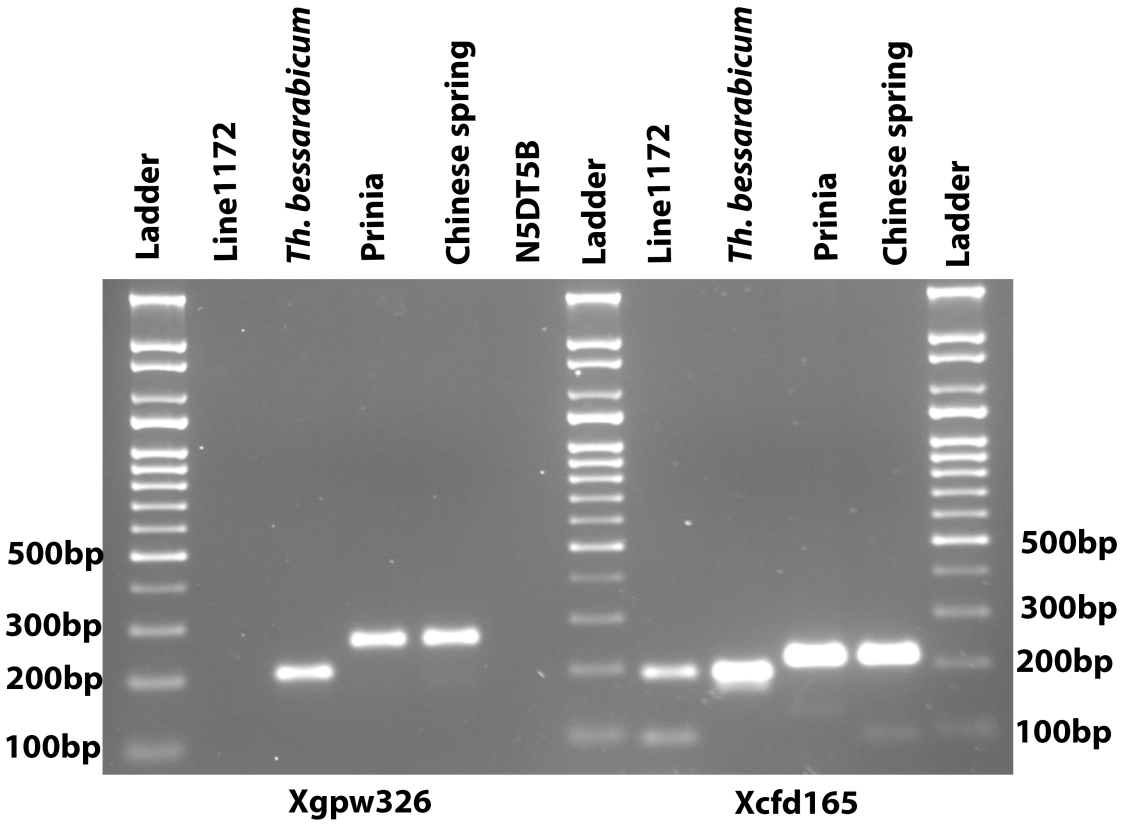


Fig. 4.7: Gel image showing PCR amplification of the wheat-*Th. bessarabicum* introgression line 1172 (T5DL.5DS-5JS) using SSR markers (a) Xgpw326 present in CS and Prinia but missing in translocation line 1172 (b) Xcfd165 present in CS and Prinia missing in translocation line 1172 and *Th. bessarabicum*.

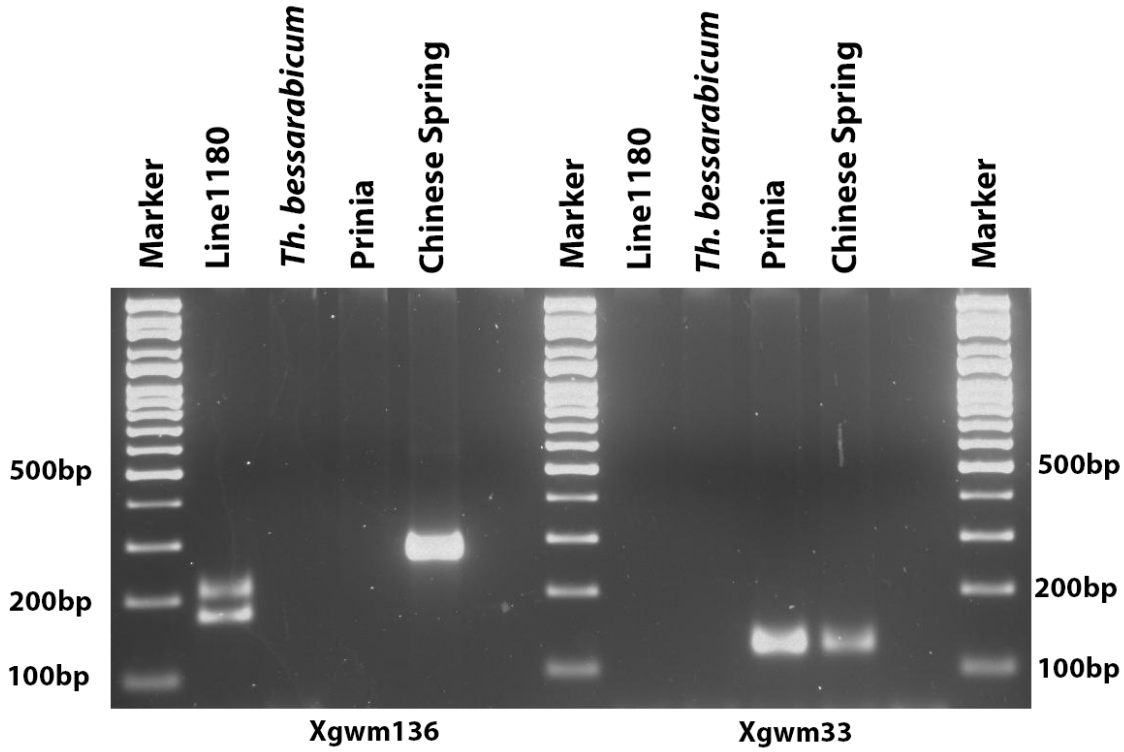


Fig. 4.8: Gel image showing PCR amplification of the wheat-*Th. bessarabicum* introgression line 1180 (T1AL-1JS) using SSR markers (a) Xgwm136 present in CS and missing in translocation line 1180 and in *Th. bessarabicum*. (b) Xgwm33 present in CS and Prinia missing in translocation line 1180 and *Th. bessarabicum*.

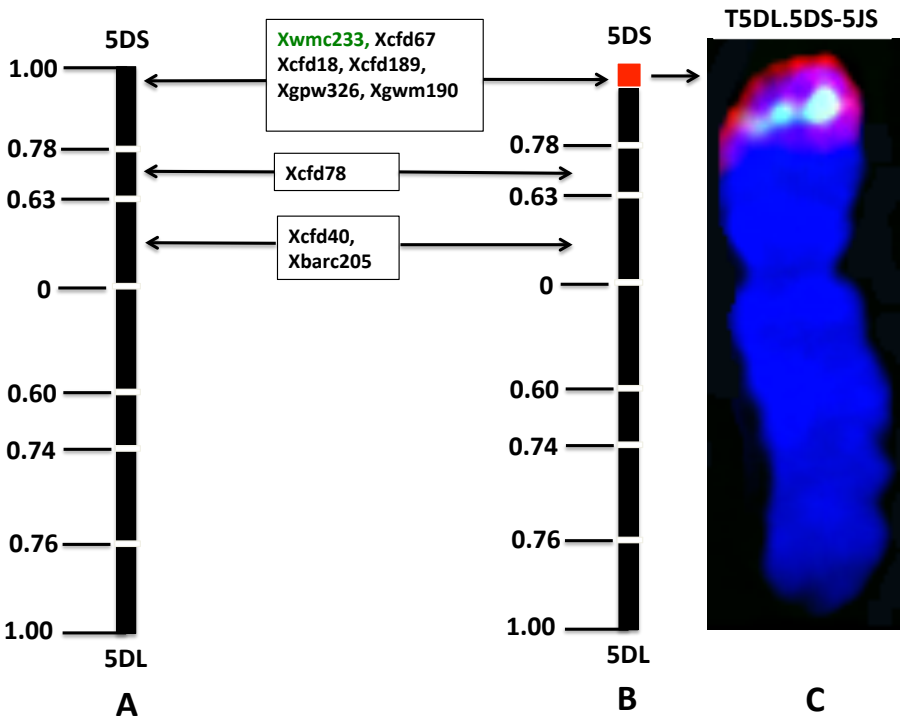


Fig. 4.9: Localisation of 5DS-specific markers on the deletion bins of deletion lines derived from Chinese Spring. Lines and the fraction lengths are indicated on the left of the schematic chromosome indicate the deletion breakpoints. Arrows indicate the positions of the markers. **B.** Physical mapping of the tested 5DS-specific markers on the terminal 5DS segment identified in the present study. Arrows indicate the positions of the markers. The marker whose position was previously unknown and which was mapped physically to the terminal region of 5DS is shown in green. The fraction length is indicated on the right side of the chromosome. **C.** FISH pattern of the line 1172 (T5DL.5DS-5JS) using *Th. bessarabicum* genomic DNA (red) and *pTa71* (yellowish green) probes.

Chapter 5: Potential for mapping novel wheat-*Th. bessarabicum* introgression lines using SNPs generated by a genotyping-by-sequencing (GBS) approach

5.1 Introduction

5.1.1 Next generation sequencing

Next generation sequencing (NGS) technologies have the potential to enable quick, inexpensive and comprehensive analysis of complex nucleic acid populations (Metzker, 2010). The first arrival of NGS, which revolutionizes genomic research, appeared in 2005 with the landmark publication of the sequencing-by-synthesis technology developed by 454 Life Sciences (Metzker, 2010, Mardis, 2011, Schuster, 2008). Sequence reads generated from NGS technologies were originally much shorter than traditional Sanger sequence reads. These technologies can generate a hundred million to billion bases data with each instrument run, which makes production, assembly and analysis of these sequence read challenging, Although several bioinformatics tools and algorithms are currently available, efforts are underway to improve the accuracy of alignment of NGS data in several laboratories (Bräutigam and Gowik, 2010, Varshney et al., 2009). The complexity, size and hexaploid nature of the wheat genome (16 Gb) makes difficult the development of molecular markers and genomic resources.

Advancements in next-generation sequencing (NGS) and related bioinformatics have provided new ways to accelerate the genetic analysis of traits. Complete and/or draft genome sequences have become available for a several cereal crop species genomes including rice, sorghum, and maize (Matsumoto et al., 2005; Paterson et al., 2009; Schnable et al., 2009). However, there are certain challenges to next-generation sequencing approaches for plant species with large and complex genomes such as barley and wheat. One main challenge is to filter and validate sets of functional genome-wide SNP markers from large sequence data sets. By using NGS, it is now possible to re-sequence a whole genome in many plant species, providing unprecedented discovery and characterization of molecular polymorphisms.

5.1.2 SNP identification using next generation sequencing (NGS)

The discovery of high-density molecular markers in crop species is now leading to a better understanding of the genetic architecture of complex traits and its application in breeding programs for crop improvement through whole genome association studies and genomic selection. The recent development of new sequencing technologies enables the discovery and detection of SNPs for plant species, where a reference genome is available (Arabidopsis) and where it is not (wheat). SNP discovery in the wheat D-genome ancestor, *Aegilops tauschii*, was recently completed using next-generation sequencing (NGS), marking a step forward for SNP markers in large and complex genomes. NGS technologies are also fast becoming the method of choice for gene expression analysis, particularly for species for which reference genome sequences are already available. Efforts are also underway to use NGS technologies for association mapping, wide crosses and alien introgression, epigenetic modifications and population biology (Tiwari et al., 2014), as a replacement for use of DNA markers. With current approaches, the complexity of the DNA to be sequenced has to be reduced considerably (to 1/10 to 1/100th of the entire genome) in order to obtain the necessary sequence redundancy for a reliable SNP calling (Ganal et al., 2009), although false discovery rates are relatively high compared to microsatellite or other PCR-based marker approaches.

The use of genome complexity reduction combined with multiplex sequencing was first demonstrated through restriction site associated DNA (RAD) tagging and NGS of the RAD tags to genetically map mutations.

5.1.3 Genotyping-by-sequencing (GBS)

Genotyping-by-sequencing (GBS) was developed by Poland et al. (2012) (collaborator in the work described here) as a simple but robust approach for complexity reduction in large complex genomes. Both RAD sequencing and GBS target the genomic sequence flanking restriction enzyme sites to produce a reduced representation of the genome. The GBS library development is greatly simplified compared to that of RAD. GBS requires less DNA, avoids random shearing and size selection, and is completed in only two steps on plates followed by PCR amplification of the pooled library. The original GBS approach used a single restriction enzyme to capture the genomic sequence between restriction sites. Here we extend the GBS protocol to a two-enzyme system that

includes one ‘‘rare-cutter’’ and one ‘‘common-cutter’’. When combined with Y-adapters for the common restriction site, the use of two enzymes differs from the original GBS protocol in that amplified fragments in the two-enzyme libraries will all consist of the barcoded forward adapter and the common reverse adapter. This type of library construction greatly simplifies quantification of the library prior to sequencing. The two-enzyme approach can generate a suitable and uniform complexity reduction. A form of this complexity reduction approach has been successfully applied in sequencing pools of BAC libraries for construction of physical maps The original GBS approach was also applied in barley to effectively map sequence tags as dominant markers on a reference map. Here, as suggested by collaborator Poland, we apply a two-enzyme GBS approach to barley and wheat and demonstrate the robustness of GBS for genotyping in species with large, complex, and even polyploid genomes. The development of high-density (10,000 to 100,000+ markers) in species that are lacking a reference genome will facilitate the development (anchoring and ordering) of the reference genome sequence while providing tools for genomics-assisted breeding.

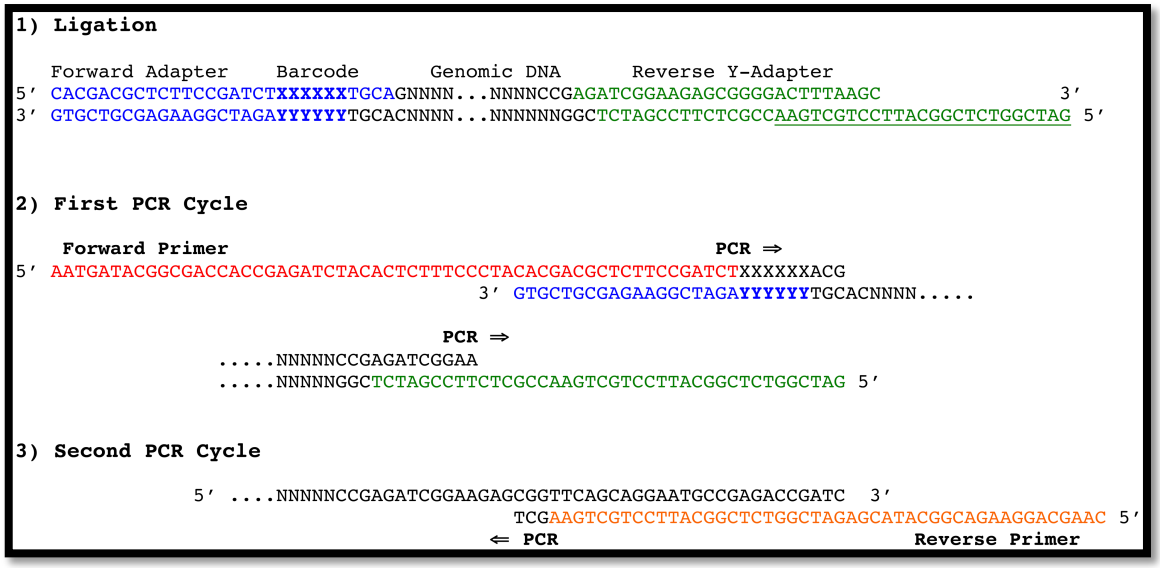


Fig. 5.1 **(1)** the ligation product of a genomic DNA fragment (black) containing a *PstI* restriction site and a *MspI* restriction site. The forward adapter (blue) binds to a *PstI* generated overhang. The 4–9 bp barcode for this adapter is in bold with “X”. The *MspI* generated overhang corresponds to the reverse Y-adapter (green). The unpaired tail of the Y-adapter is underlined. **(2)** During the first round of PCR only the forward primer (red) can anneal. PCR synthesis of the complementary strand proceeds to the end of the fragment synthesizing the compliment of the Y-adapter tail. **(3)** During

the second round of PCR the reverse primer (orange) can anneal to the newly synthesized compliment of the Y-adapter tail. This PCR reaction then proceeds to fill in the complement of the forward adapter/primer on the other end of the same fragment (Poland et al., 2012).

5.1.4 Aims

Many researchers have aimed to identify breakpoints involved in translocations (Tiwari et al., 2014), and to exploit alien introgressions without requiring extensive cytogenetics and marker work. Here, we aimed to test a massively parallel sequencing approach with the six novel wheat-*Thinopyrum bessarabicum* recombinant lines, which were already characterized (Chapter 3 and Chapter 4) in order to validate the GBS approach

5.2 Material and Methods

5.2.1 Genomic DNA extraction

Genomic DNA extracted from leaf tissue as explained in Chapter 2 Materials and Methods.

5.2.2 GBS sequencing and data analysis

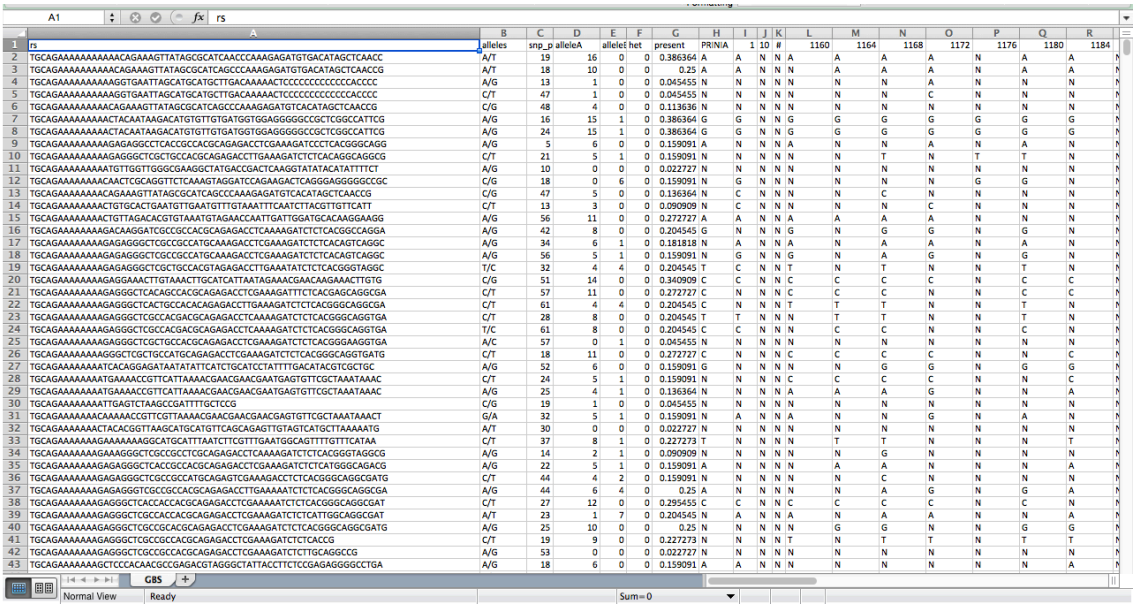
The DNA samples were sent to Jesse Poland's lab at Kansas State University (KSU) to run GBS. The data were received and processed through GBS pipeline (Glaubitz et al., 2014).

5.3 Results

5.3.1 Mapping of SNPs

After receiving data from 6 lines in Hap Map file, it was converted into the tab-delimited format (Fig. 5.2). The first column contains genotypes, second column= SNP position, Third column= allele A, fourth column= allele B, fifth column= allele frequency, rest of the column contains lines and SNPs. N denotes missing data (Fig. 5.2). After SNP calling we found 48,000 genotypes, after mapping them with POPSEQ mapping (Chapman et al., 2015) (Fig. 5.3), only 18,127 markers have been mapped to a chromosome (only 16,675 have a mapping quality of 20+). These markers were not

enough to map small introgressions. Although GBS has been widely used in population diversity study and association mapping, use of GBS for identifying wheat-alien introgression is very limited. To analyze this large and unambiguous dataset we need to develop sophisticated bioinformatic pipelines to identify small alien introgressions in wheat.



rs	alleles	snp_p	alleleA	allele	het	present	PRINA	1	10	#	1160	1164	1168	1172	1176	1180	1184
TGCAGAAAAAAAAAAACAGAAAGTTATAGCGCATCAACCCAAAGAGATGTGACATAGCTCAACC	A/T	19	16	0	0	0.386364	A	A	N	N	A	A	A	A	N	A	A
TGCAGAAAAAAAAAACAGAAAGTTATAGCGCATCAGCCCAAAGAGATGTGACATAGCTCAACCG	A/T	18	10	0	0	0.25	A	A	N	N	N	A	A	A	N	A	A
TGCAGAAAAAAAAAAGGTGAATTAGCATGCATGCTTGACAAAAACTCCCCCCCCCCCCCCACCC	A/G	13	1	0	0	0.045455	N	N	N	N	N	N	A	N	N	N	N
TGCAGAAAAAAAAAGGTGAATTAGCATGCATGCTTGACAAAAACTCCCCCCCCCCCCCCACCC	C/T	47	1	0	0	0.045455	N	N	N	N	N	N	N	C	N	N	N
TGCAGAAAAAAAACAGAAAGTTATAGCGCATCAGCCCAAAGAGATGTCACATAGCTCAACCG	C/G	48	4	0	0	0.113636	N	N	N	N	N	N	N	N	N	N	N
TGCAGAAAAAAAACTACAATAAGACATGTGTTGTGATGGTGGAGGGGGCCGCTCGGCCATTCG	A/G	16	15	1	0	0.386364	G	G	N	N	G	G	G	G	G	G	G
TGCAGAAAAAAAACTACAATAAGACATGTGTTGTGATGGTGGAGGGGGCCGCTCGGCCATTCG	A/G	24	15	1	0	0.386364	G	G	N	N	G	G	G	G	G	G	G
TGCAGAAAAAAAAAGAGAGGCCTCACCGCCACGCAGAGACCTCGAAAGATCCCTCACGGGCAGG	A/G	5	6	0	0	0.159091	A	N	N	N	A	N	N	A	N	A	N
TGCAGAAAAAAAAAGAGGGCTCGCTGCCACGCAGAGACCTTGAAAGATCTCTCACAGGCAGGCG	C/T	21	5	1	0	0.159091	N	N	N	N	N	T	N	T	T	T	N
TGCAGAAAAAAAAAATGTTGGTTGGGCGAAGGCTATGACCGACTCAAGGTATATACATATTTTCT	A/G	10	0	0	0	0.022727	N	N	N	N	N	N	N	N	N	N	N
TGCAGAAAAAAAACAACTCGCAGGTTCTCAAAGTAGGATCCAGAAGACTCAGGGAGGGGGCCGC	C/G	18	0	6	0	0.159091	N	G	N	N	N	N	N	G	G	N	N
TGCAGAAAAAAAACAGAAAGTTATAGCGCATCAGCCCAAAGAGATGTCACATAGCTCAACCG	C/G	47	5	0	0	0.136364	N	C	N	N	N	C	N	N	N	N	N
TGCAGAAAAAAACTGTGCACTGAATGTTGAATGTTTGTAAATTTCAATCTTACGTTGTTCATT	C/T	13	3	0	0	0.090909	N	C	N	N	N	N	C	N	N	N	N
TGCAGAAAAAAAACTGTTAGACACGTGTAAATGTAGAACCAATTGATTGGATGCACAAGGAAGG	A/G	56	11	0	0	0.272727	A	A	N	N	A	A	A	N	N	N	N
TGCAGAAAAAAAAGACAAGGATCGCCGCCACGCAGAGACCTCAAAGATCTCTCACGGCCAGGA	A/G	42	8	0	0	0.204545	G	A	N	N	G	N	G	G	N	G	N
TGCAGAAAAAAAAGAGAGGGCTCGCCGCCATGCAAAGACCTCGAAAGATCTCTCACAGTCAGGC	A/G	34	6	1	0	0.181818	N	A	N	N	A	A	A	N	A	A	N
TGCAGAAAAAAAAGAGAGGGCTCGCCGCCATGCAAAGACCTCGAAAGATCTCTCACAGTCAGGC	A/G	56	5	1	0	0.159091	N	G	N	N	G	N	A	G	N	G	N
TGCAGAAAAAAAAGAGAGGGCTCGCTGCCACGTAGAGACCTTGAAATATCTCTCACGGGTAGGC	T/C	32	4	4	0	0.204545	T	C	N	N	T	N	T	N	N	T	N
TGCAGAAAAAAAAGAGGAAACTTGTAAACTTGCATCATTAATAGAAACGAACAAGAAACTTGTG	C/G	51	14	0	0	0.340909	C	C	N	N	C	C	C	N	N	C	C
TGCAGAAAAAAAAGAGGGCTCACAGCCACGCAGAGACCTCGAAAGATTTCTCACGAGCAGGCGA	C/T	57	11	0	0	0.272727	C	N	N	N	C	C	C	N	N	C	C
TGCAGAAAAAAAAGAGGGCTCACTGCCACACAGAGACCTTGAAAGATCTCTCACGGGCAGGCGA	C/T	61	4	4	0	0.204545	C	N	N	N	T	T	T	N	N	T	N
TGCAGAAAAAAAAGAGGGCTCGCCACGACGCAGAGACCTCAAAGATCTCTCACGGGCAGGTGA	C/T	28	8	0	0	0.204545	T	T	N	N	N	T	T	N	N	T	N
TGCAGAAAAAAAAGAGGGCTCGCCACGACGCAGAGACCTCAAAGATCTCTCACGGGCAGGTGA	T/C	61	8	0	0	0.204545	C	C	N	N	N	C	C	N	N	C	N
TGCAGAAAAAAAAGAGGGCTCGCTGCCACGCAGAGACCTCGAAAGATCTCTCACGGGAAGGTGA	A/C	57	0	1	0	0.045455	N	N	N	N	N	N	N	N	N	N	N
TGCAGAAAAAAAAGGGCTCGCTGCCATGCAGAGACCTCGAAAGATCTCTCACGGGCAGGTGATG	C/T	18	11	0	0	0.272727	C	N	N	N	C	C	C	N	N	C	C
TGCAGAAAAAAAATCACAGGAGATAATATATTCATCTGCATCCTATTTTGACATACGTCGCTGC	A/G	52	6	0	0	0.159091	G	N	N	N	N	G	G	N	N	G	G
TGCAGAAAAAAATGAAAACCGTTCATTAAAACGAACGAACGAATGAGTGTTCGCTAAATAAAC	C/T	24	5	1	0	0.159091	N	N	N	N	C	C	C	N	N	N	C
TGCAGAAAAAAAATGAAAACCGTTCATTAAAACGAACGAACGAATGAGTGTTCGCTAAATAAAC	A/G	25	4	1	0	0.136364	N	N	N	N	A	A	A	G	N	N	A
TGCAGAAAAAAAATTGAGTCTAAGCCGATTTTGCTCCG	C/G	19	1	0	0	0.045455	N	N	N	N	N	N	N	N	N	N	N
TGCAGAAAAAAACAAAAACCGTTCGTTAAAACGAACGAACGAACGAGTGTTCGCTAAATAAACT	G/A	32	5	1	0	0.159091	N	A	N	N	A	N	N	G	N	A	N
TGCAGAAAAAACTACACGGTTAAGCATGCATGTTCAGCAGAGTTGTAGTCATGCTTAAAAATG	A/T	30	0	0	0	0.022727	N	N	N	N	N	N	N	N	N	N	N
TGCAGAAAAAAAAGAAAAAAAGGCATGCATTTAATCTTCGTTTGAATGGCAGTTTTGTTTCATAA	C/T	37	8	1	0	0.227273	T	N	N	N	T	T	N	N	N	N	N
TGCAGAAAAAAAGAAAGGGCTCGCCGCCTCGCAGAGACCTCAAAAGATCTCTCACGGGTAGGCG	A/G	14	2	1	0	0.090909	N	N	N	N	N	G	N	N	N	N	N
TGCAGAAAAAAAAGAGAGGGCTCACCGCCACGCAGAGACCTCGAAAGATCTCTCATGGGCAGACG	A/G	22	5	1	0	0.159091	A	N	N	N	A	A	N	N	N	N	A
TGCAGAAAAAAAAGAGAGGGCTCGCCGCCATGCAGAGTCGAAAGACCTCTCACGGGCAGGCGATG	C/T	44	4	2	0	0.159091	N	N	N	N	N	C	N	N	N	N	N
TGCAGAAAAAAAAGAGAGGGTCGCCGCCACGCAGAGACCTTGAAAATCTCTCACGGGCAGGCGA	A/G	44	6	4	0	0.25	A	N	N	N	N	A	G	N	N	G	A
TGCAGAAAAAAAAGAGGGCTCACCACACGCAGAGACCTCGAAAAATCTCTCACGGGCAGGCGAT	C/T	27	12	0	0	0.295455	C	C	N	N	C	C	C	N	N	C	C
TGCAGAAAAAAAAGAGGGCTCGCCACCACGCAGAGACCTCGAAAGATCTCTCATTGGCAGGCGAT	A/T	23	1	7	0	0.204545	N	A	N	N	A	N	A	A	N	N	A
TGCAGAAAAAAAAGAGGGCTCGCCGCACGCAGAGACCTCGAAAGATCTCTCACGGGCAGGCGATG	A/G	25	10	0	0	0.25	N	N	N	N	G	G	N	N	G	G	G
TGCAGAAAAAAAAGAGGGCTCGCCGCCACGCAGAGACCTCGAAAGATCTCTCACAGTCAGGCGA	C/T	19	9	0	0	0.227273	N	N	N	N	T	N	T	T	N	T	T
TGCAGAAAAAAAAGAGGGCTCGCCGCCACGCAGAGACCTCGAAAGATCTCTTGCAGGCCG	A/G	53	0	0	0	0.022727	N	N	N	N	N	N	N	N	N	N	N
TGCAGAAAAAAAGCTCCCACAACGCCGAGACGTAGGGCTATTACCTTCTCCGAGAGGGGCCTGA	A/G	18	6	0	0	0.159091	A	A	N	N	N	N	N	N	N	N	A

Fig. 5.2: Screenshot of GBS data in XL file.

contig	length	chr	cM	MAPQ	rs	alleles	snp_pos	alleleA	alleleB	het	present	PRINIA	X1	X10	X11	X1160	X1164	X1168
ta_contig_1A	3242	1A	0	37	TGCAGCGCT	C/T	31	0	1	0	0.04545455	N	N	N	N	N	N	N
ta_contig_1A	3242	1A	0	37	TGCAGCGCT	C/T	51	0	1	0	0.04545455	N	N	N	N	N	N	N
ta_contig_1A	12068	1A	0	25	TGCAGCTAT/	A/G	7	10	1	0	0.27272727	N	N	N	N	N	A	A
ta_contig_1A	12068	1A	0	25	TGCAGCTAT/	A/G	47	9	1	0	0.25	N	N	N	N	N	G	G
ta_contig_1A	1316	1A	0	25	TGCAGCTCG	A/G	38	9	1	0	0.25	G	N	N	N	G	N	N
ta_contig_1A	1316	1A	0	25	TGCAGCTCG	C/G	40	4	6	0	0.25	C	N	N	N	C	N	N
ta_contig_1A	1776	1A	0	37	TGCAGCTTTT	A/T	58	1	3	0	0.11363636	A	N	N	N	N	N	T
ta_contig_1A	1776	1A	0	37	TGCAGCTTTT	G/A	60	1	4	0	0.13636364	G	N	N	N	N	A	A
ta_contig_1A	3234	1A	0	37	TGCAGGTTT(	C/T	17	4	0	0	0.11363636	N	N	N	N	T	N	N
ta_contig_1A	3234	1A	0	37	TGCAGGTTT(	G/C	36	4	0	0	0.11363636	N	N	N	N	C	N	N
ta_contig_1A	6468	1A	0	37	TGCAGTGTT	C/T	17	2	0	0	0.06818182	N	N	N	N	N	C	N
ta_contig_1A	6468	1A	0	37	TGCAGTGTT	C/A	29	1	0	0	0.04545455	N	N	N	N	N	C	N
ta_contig_1A	16381	1A	0	25	TGCAGTTCG(	C/T	7	0	1	0	0.04545455	N	N	N	N	N	N	N
ta_contig_1A	4988	1A	3.889	25	TGCAGGTGC	A/C	51	4	5	0	0.22727273	C	N	N	N	C	C	N
ta_contig_5(	3804	1A	4.226	0	TGCAGCTTC(	C/G	43	5	0	0	0.13636364	N	N	N	N	N	N	N
ta_contig_1A	9478	1A	4.562	25	TGCAGCAAA	A/G	18	9	0	0	0.22727273	G	N	N	N	G	N	N
ta_contig_1A	9478	1A	4.562	25	TGCAGCAAA	C/T	51	7	5	1	0.31818182	C	N	N	N	C	T	T
ta_contig_1A	7445	1A	4.562	37	TGCAGCAAT/	A/C	21	0	3	0	0.09090909	N	N	N	N	N	N	C

Fig. 5.3: Screenshot of GBS data file showing chromosomal location of SNPs after POPSEQ mapping.

5.4 Discussion

It is clear that GBS is going to become a critical technology replacing others of markers. However, the approaches are still at early stages of development and both technology and analytical methods will be rapidly changing in a monthly timescale. Here, data were generated for an early look at the potential and current value of this approach.

As GBS data are sparse and prone to error (Spindel et al. 2013), in our results here, we could able to map 18,127 markers, as the introgressions were small these markers weren't enough to map them. The size of our population was small. We needed big population size for introgression mapping to validate data. Our study shows that, to map alien introgression using GBS we need to have enough population size and good choice of markers to capture diversity from introgress regions, so under the right circumstances GBS can fulfill this hope.

A disadvantage with GBS information received here was the level of missing data, presumably mostly because of technical issues like PCR amplification bias during the library construction step and leading to not all sequenced regions of interest being evenly covered in all individuals within a population. Data analysis and insufficiently sophisticated bioinformatic tools available is one biggest barrier to the widespread use of GBS as is accompanied by a high number of erroneous SNP calls which are difficult to detected or corrected. However, to address this issue several bioinformatics pipelines have been developed (Bradbury et al., 2007, Spindel et al., 2013, Sonah et al., 2013). It is necessary to generate high-density SNP datasets in fixed collections of introgression lines, which makes possible to define the size and positions of both target and background introgressions and to identify small donor introgressions that had been missed using previous, lower density marker datasets.

SNP detection in wheat, as in barley, is a challenging task because the very large genome sizes (16 Gb for hexaploid wheat vs. 0.13 Gb for Arabidopsis) require reduced-representation strategy for sequencing. Often with reduced-representation, it is hard to obtain suffcicient presentation of the genome and hence reduces the chance to capture more markers and diversity from genomes.

Detecting SNP within alien introgression is often difficult with conventional PCR markers, as shown in Chapter 4. With GBS, by early 2015, no studies have analyzed alien-wheat lines to characterize the size and location of introgressed chromosome

segments in the way we aimed to achieve here. With the GBS data here, limited analysis using current bioinformatic tools couldn't map SNPs in introgressed regions. There are two possible explanations for this: one might not have captured markers from introgressed regions, second the choice of restriction enzyme used to perform GBS. Tiwari et al. (2014) mapped wheat-*Ae. geniculata* alien introgression lines using next generation sequencing approach by flow sorting and sequencing individual introgress chromosome. A similar kind of approach would have worked here to map the wheat-*Thinopyrum* translocation lines, but flow-sorting is a complex technique and certainly not of universal applicability as is the hope for GBS genotyping approaches.

Chapter 6: General conclusion

These general conclusions reflect on the aims presented in Chapter 1 introduction and addressed in the three experimental chapters of this thesis, which described a molecular (DNA) and molecular cytogenetic approach to study and characterize the wheat-*Th. bessarabicum* introgression lines. The reported results in different chapters (Chapters 3-5) on wheat-*Th. bessarabicum* hybrids can lay the foundation for more rational approaches for molecular mapping and introgression of important traits into wheat. In this context, the following topics will be discussed in more detail in subsequent paragraphs to draw attention to the practical and theoretical aspects of introgression of alien segments into wheat.

6.1 Exploitation of the *Thinopyrum* genus as a potential source of useful genes

As discussed in Chapter 1 and Chapter 3, the introduction of alien genetic variation, in particular of species belonging to *Thinopyrum* genus (tertiary gene pool), through chromosome engineering into wheat is a valuable and proven technique for wheat improvement (Sears, 1977, Gale and Miller, 1987, Chen, 2005). Many different species of *Thinopyrum* have been crossed with wheat, but the greatest success has been obtained with diploid *Th. elongatum* (Fu et al., 2012, Hu et al., 2012), hexaploid *Th. intermedium* (Graybosch et al., 2009, Mutti et al., 2011) and decaploid *Th. ponticum* (Sepsi et al., 2008). However, the work on *Th. bessarabicum* (2*n*=2*x*=14, JJ genome) has been more limited (King et al., 1997, William and Mujeeb-Kazi, 1993). After the success of Sears (1956, 1977) first transfer of *Lr19* genes from *Aegilops umbellulata* to wheat, breeders have successfully developed several wheat-*Thinopyrum* hybrids to transfer resistance genes into bread wheat. Earlier studies have revealed about 18 novel disease resistance genes introgressed from *Thinopyrum* species to bread wheat using both irradiation treatment and homoeologous recombination (Fedak & Han 2005).

The work presented here aimed to identify and characterize six novel wheat-*Th. bessarabicum* translocation lines using both cytogenetic (Chapter 3) and molecular marker approaches (Chapter 4). We demonstrated that molecular cytogenetic techniques such as GISH, FISH, and the combination when used in physical mapping procedures provide an important and effective tool to investigate and characterize the alien introgressions. The uses of repetitive DNA probes that have characteristic banding

patterns for most wheat chromosomes (Chapter 3) were very effective in identifying the recipient wheat chromosome (Chapter 3). The combination of molecular cytogenetic analysis used in this study was effective in characterizing the wheat-*Th. bessarabicum* recombinants (Chapter 3). The result of the simultaneous GISH and FISH analysis indicated that D-genome of wheat is very close to *Th. bessarabicum* genome (Chapter 3). Similar evidence was presented by Liu et al. (2007) who found that E genomes DNA readily hybridized to wheat D genome compared to the A and B genomes, indicating the close affinity of the E genome to the D genome, which contradicted Riley et al. (1958) findings where he considered both cytogenetic evidence and other researches involving hybrids of polyploid *Agropyrons* with tetraploid and hexaploid wheat. Since there has apparently been no character transfer from *Agropyron* to wheat, he stated, "The bulk of the evidence indicates that there is no genome in *Agropyron* closely related to a wheat genome."

Induction of homoeologous pairing for producing wheat-alien translocation, which can import alien chromosome segments or useful genes of the wild relatives into recipient wheat, has been a popular choice since last decade. Since then more efforts have been made to target the wheat genetic stocks using cytogenetic manipulation systems to promote homeologous exchanges. It has been used to transfer gene-conditioning resistance to the leaf rust (*Lr24*) from two different *Agropyron elongatum* chromosomes to wheat chromosomes (Riley et al. 1968; Sears 1972). The preferred options for encouraging the frequency of homoeologous chromosome pairing and recombination are the use of *Ph1* (Chen et al., 1994) or the *ph1b* (Sears, 1977, 1981, 1982) stocks, which later can produce genetically compensating translocations (Niu et al., 2011, Qi et al., 2008). The *Ph* wheat-mutant system has a long history of successful use in generating recombinants (Sears, 1953, Riley and Chapman, 1958).

The lines used in this study, made through CIMMYT, have been based on the latter and exploited the CS/*Th. bessarabicum* amphiploid combination that is *PhPh* in its genetic control structure (Chapter 3). Chapter 3 demonstrates a standard approach in which a donor alien chromosome placed in a suitable genetic background is identified, combined with the *ph1b* mutation or a system suppressing the *Ph1* locus, and recombinant wheat-alien chromosomes are recovered (Sears 1981). The advantage of induced homoeologous pairing is the compensating transfer of desired alien chromosome segments reducing the unwanted alien chromatin (Baum et al., 1992, Dyck

et al., 1990). Molnar-Lang et al. (2000) produced wheat–barley translocations at a higher frequency (20%) as an effect of in vitro culture.

These compensating translocations can then be engineered to generate more desirable small intercalary alien translocations in wheat chromosomes. However, the frequency of homoeologous recombination depends upon various factors such as the level of affinity between the donor alien and the recipient wheat chromosome. The size of populations needed to generate and identify the primary wheat-alien chromosome recombinants can be very large (Lukaszewski 2000) and difficult to screen. The structurally rearranged segments of alien chromosomes, genetic differences between the donor and recipient chromosomes and the genetic distance between the target gene and the centromere play a vital role (Nasuda et al., 1998, Qi et al., 2007, Lukaszewski, 2001). Sequence divergence between alien and donor is one of them which could result in reduced recombination, Canady et al. (2006) detected a genome-wide reduction in recombination frequencies within introgressed *S. lycopersicoides* segments, frequently low as 0–10% of normal levels.

The recombination between wheat-*Thinopyrum* chromosomes has been extensively studied before. *Thinopyrum* chromosome of Triticeae group 6 paired in 4.8% of the meiotic cells in the presence of the *Aegilops speltoides* genome, which resulted in the suppression of the *Ph1* locus (Johnson and Kimber, 1967). The relative transfer rates of the alien chromosome in wheat was 6.7 and 8.7% for *Th. ponticum* (3Ag, 7Ag chromosomes) carrying leaf rust resistance (Sears, 1973, Sears, 1983). Allosyndetic recombinations between the 7Ai chromosome of *Th. intermedium* and wheat homoeologue have been reported to be 16% by Khan (1999). The pairing between wheat and 6Ae chromosome of diploid *TH. elongatum* was found to be 4.4% of the sporocytes, whereas it was 2.48% for the 6Ag chromosome (*Th. ponticum*) with wheat chromosome 6D when both were in the monosomic condition (Dvořák, 1979, Yasumuro et al., 1981).

The success of chromosome engineering for targeted introgression of alien genes is dependent on the elimination of the deleterious effects of the introgressed alien chromatin in the crop plant. However, producing intercalary translocations is the best way to achieve small alien segment with reduced linkage drag, for example, reducing large amount of *Aegilops speltoides* chromatin surrounding *Sr39* (Niu et al., 2011), a reduced *Haynaldia villosa* containing chromatin the *Pm21* locus for powdery mildew

resistance in wheat (Chen et al., 2013), and the development of wheat-rye terminal and intercalary chromosomal translocations (Lukaszewski, 2000). Intercalary translocation has proven to have potential applications, Zhang et al. (2015) developed Ti1AS-6PL-1AS·1AL intercalary translocation line carrying *Agropyrum cristatum* 6P chromosome segment showed a enhance grain weight and spike length. Chen et al. (1995) produced wheat-*H. villosa* 6VS/6AL translocation lines specifying resistance to powdery mildew. Friebe et al. (1991) transferred Hessian fly resistance from rye to wheat via radiation-induced terminal and intercalary chromosomal translocations.

Additionally, all the translocation lines studied here were fertile with good morphological characteristics and hence very useful in breeding programme. The research work carried out here demonstrates that potential genes from *Th. bessarabicum* could be transferred to wheat, through gene introgression and the production of new wheat-*Th. bessarabicum* hybrids. Knowledge on the cytogenetic background of the genetic material used in wheat improvement is crucial for designing modern breeding programs. The exploration of the intergenomic rearrangements occurring in wheat-alien amphiploids using *in situ* hybridization and molecular markers will facilitate the selection of progenies carrying the chromosome segments associated with agronomically important genes. Such studies also provide a better understanding of the process of alien introgression, revealing the effects of various introgressions and rearrangements in the genetic background of wheat.

6.2 Breeding using wheat x alien hybrids

Introgressive hybridization is a commonly practiced strategy for incorporating valuable traits from related species via interspecific or intergeneric hybridization and repeated backcrossing and has been widely used by plant breeders (Anderson, 1953). Wheat-alien hybridization is excellent a way to transfer agronomically useful genes from one species to the other (Chapter 3). Several useful alien gene transfers have been reported from closely related wild species or rye (*Secale cereale*) into wheat, but very few works has been reported from barley (*Hordeum vulgare*) (Molnár-Láng et al., 2014), Many more genes have been transferred from the *Thinopyrum* genus (Ali, 2012, Patokar et al., 2015 In press). Resistance has been introgressed into wheat from at least 52 species from 13 genera (Wulf and Moscou, 2014). Intergeneric hybrids between wheat and related species are generally almost completely sterile. The use of colchicine for the

production of amphiploid via the doubling of chromosome of such hybrids increases the prospects to obtain fertile plants with a stable genetic background, including the chromosome sets of both parents. This amphiploid is then used in subsequent crosses to produce wheat-alien addition lines following O'mara (1940) method used in the production of wheat-rye lines. The lines used in this study followed the similar method for production (Chapter 3).

Addition lines were considered as a useful way for mapping and determining the gene content. However, all attempts to produce amphiploid from barley x wheat F1 hybrids failed, but some 49-chromosome progeny (heptaploids) were obtained after backcrossing them with wheat pollen (Islam and Shepherd, 1992). Some of the *Hordeum chilense*-wheat hybrids have been more successful and also Tritordeum amphiploids have been made as fertile hybrid derivatives (Martın et al., 1999, Martín et al., 1995, Martín et al., 1998). Islam et al. (2007) produced *H. marinum* x wheat amphiploids. The present study emphasizes on exploiting wild species *Th. bessarabicum* for wheat genetic improvement (Chapter 3). We have successfully demonstrated that wheat-*Th. bessarabicum* amphiploid was really useful in karyotyping and characterizing all seven J-genome chromosomes (Chapter 3, Section-II).

6.3 Molecular markers detect chromosome introgression but not translocations

In bread wheat, marker availability and recombination rate hamper the cloning and characterization of introgressed genes in the hexaploid with large genomes (as discussed in Chapter 4). While NGS technologies like GBS can now solve the problem of developing markers (Chapter 5), such map-based approximation approaches can face difficulty due to a lack of recombination. Most of the grass genomes have suppressed recombination, linkage disequilibrium or segregation distortion (e.g. Anhalt et al. (2008). Recently in wheat it was found that all crossover events on chromosome 3B occurred in only 13% of the chromosome (Choulet et al., 2014) while in barley 50% of the recombination occurs in 5% of the genome (Künzel et al., 2000). Even where recombination does happen, it is usually uneven particularly between wheat and alien chromatin (Qi et al., 2007), and the resolution is not high enough to directly identify the gene/alien chromatin with SNP or other molecular markers (Wulff and Moscou, 2014).

Generally chromosome manipulation by homoeologous recombination has been restricted to a single chromosome or chromosome arm (Qi et al., 2007). The screening of homeologous recombinants should be complemented with DNA markers. However, with the help of DNA marker analysis it has been possible to produce and screen genome-wide recombinant chromosome stocks. Islam and Shepherd (1992) obtained wheat–barley recombinants involving the chromosome arms 3HL and 6HL at frequencies of 1.1% and 1.4%, respectively, using the *Ph1b* mutation and isozyme markers. Qi et al. (2007) proposed the scheme, based on the fact that homoeologous recombination is limited to one or a few sites in each arm and genes determining most agronomic traits are located in the terminal ends of chromosomes (Heslop-Harrison, 1991). In addition the number of progenies is needed for the whole genome is same as is needed for a single chromosome arm manipulation scheme, to achieve this a battery of PCR-based co-dominant centromeric and telomeric markers are required (Qi et al., 2007).

The knowledge on *Th. bessarabicum* specific DNA markers that could be used to document homoeologous recombination is poor. With the help of published marker microsatellite marker (SSR), we were able to map wheat-alien translocation (Chapter 4). Other methods for introgression detection include genotyping by sequencing (GBS) (Chapter 5), which generate restriction fragments that can be subsequently sequenced for later SNP calling (Poland et al., 2012, Arbelaez et al., 2015, Baird et al., 2008).

GBS allows high-throughput detection of thousands of SNPs along the genome. Since it utilizes the very low coverage of NGS reads, it can accommodate multiple samples and can cover everything from a single gene polymorphism to a whole genome resequence. It can produce a higher polymorphism than other conventional PCR-based markers. Considering its low cost compared to other sequencing technologies; undoubtedly GBS is a rapidly developing field. Resolution can depend on even coverage and marker distribution along chromosomes: to gain cost-effective and analyzable amounts of data, current techniques use relatively rare-cutting enzymes, which are not even over the chromosomes. GBS low coverage often results in a number of genotyping errors. Sometimes, sequencing errors also result in false genotypes. As it is based on restriction enzymes, it has been noticed that there is a high rate of non-calls and relatively low reproducibility that makes GBS less suitable for introgression detection (Chapter 5) (Galvão et al., 2012). Despite the remaining challenges, analysis

developments are likely to make GBS the fascinating choice for all high-value breeding programs in the next few years (Kim et al., 2015).

6.4 Advantages of molecular cytogenetics investigations

Molecular cytological techniques (GISH, FISH and C-banding) remains an excellent starting point for analyzing genomes of wheat x alien hybrids, defining and characterizing wheat-alien translocations and karyotypic analysis (Molnár-Láng et al., 2000, Linc et al., 2011, Sepsi et al., 2008, Schwarzacher et al., 1989). This techniques, helps us to study the molecular composition of chromosomes, and has extended our ability to identify specific chromosomes, distribution of repetitive DNA families, large fragment clones (BAC-FISH) and small oligonucleotides (Cuadrado et al., 2000, Linc et al., 2011). Along with wheat molecular cytogenetics has been used and popular among other crops as well for example, Dong et al. (2001) revealed the genetic identity of alien chromosomes and segments in potato breeding lines using combined chromosome painting with GISH and BAC-FISH. In *Brassicaceae*, FISH has been used for examining alien introgression including important fertility restoration genes from *Raphanus* into *Brassica rapa* to assist making F1 hybrid seed (Niemelä et al., 2013) and extensively for comparative genomics (Lysak et al., 2005).

Here, chapter 3 and chapter 4 shown that *in situ* hybridization is also the most efficient and accurate technique for allocating the breakpoints and to estimate the chromatin size in the translocation chromosomes (Le et al., 1989; Schwarzacher et al., 1989; Jiang and Gill, 1994). The detailed characterization has been shown here in a majority of our work (Chapter 3, Section 1, 2); wherein specific probes have been successful in the identification of specific genomes and chromosomal regions. Based on the localization of the different sequences on chromosomes, we can construct the wheat-alien cytogenetic maps, providing a framework for structural and functional genomics research. *In situ* hybridization can also detected alien chromosomes in late breeding lines, e.g. in a BC6 line of *Pennisetum squamulatum* with a *P. glaucum* introgression (Goel et al., 2003). In the assembly of the tomato genome, based on mapping using a cross with distant parents, *in situ* hybridization was essential to define the arrangement of sequence scaffolds (Shearer et al., 2014) who concluded that “similar errors exist in pseudomolecules from other large genomes that have been assembled using only

linkage maps to predict scaffold arrangement, and these errors can be corrected using FISH".

But cytogenetic experiments require high expertise and involve intact nuclei and samples must be processed within a short amount of time. A long time makes the cytogenetic approach expensive when there is a need of analyzing hundreds of plants. Thus, breeders must be provided with methods of assessment rapid and that can be routinely applied when large numbers of plants have to be screened. Nevertheless, although an individual line can be characterized, but throughput is usually low and each line will require two weeks or more of full-time work for characterization by molecular cytogenetics.

6.5 Next generation sequencing and crop genomics

The analysis and detection of genome-wide single nucleotide polymorphisms (SNPs) emerge as an excellent strategy that becomes increasingly popular and attractive for deciphering genome organization and topological context of target genes underlying agronomically important traits. High-throughput and ultradense NGS-genotyping such as GBS (Genotyping-by-sequencing) will help in the production of wheat–alien introgression lines (ILs) with high background isogenicity (Reynolds et al., 2012). Recently SNP discovery in the wheat D-genome predecessor, *Aegilops tauschii*, was completed using next-generation sequencing (NGS), marking a step forward for SNP markers in large and complex genomes (You et al., 2011). In maize 56,000 SNP markers were derived from the comparison of the B73 maize reference genome using Illumina Infinium MaizeSNP50 chip (Deschamps et al., 2012).

SNP discovery in a complex crop like wheat is challenging due to (1) its repetitive nature and hexploidy, the existence of homeologous sub-genomes sharing ~96%±98% identities in tetraploid or hexaploid wheat easily confound SNP detection (2) absence of a reference genome sequence, (3) availability of gene sequences from only a few genotypes, (4) low polymorphism levels due to a reduced nucleotide diversity of the wheat genome the existence of polymorphisms between them, known as inter-homoeologue polymorphism (IHP) (Koebner and Summers 2002; Edwards and Batley 2010). However, several successful attempts for SNP discovery in wheat were made and many more underway (Trebbi et al., 2011, Trick et al., 2012). Recent advancement in NGS platforms gives rise to the generation of large amounts of DNA information in a

very short period of time. The analysis of these large amount of data requires powerful computers and complex algorithms and have led to a recent growth of the bioinformatics field of research (Kumar et al., 2012, Shendure et al., 2008).

Several bioinformatics software packages such as introgression browser (iBROWSER) are available to visualize such whole-genome SNP (wgSNP) data. These softwares are effective to describe and map introgressed segments, identifies donor parents, and is able deal with large number of genomes with practically no genome size constraint (Posada 2002; Martin et al. 2011; Lechat et al. 2013; Kim et al. 2015; Aflitos et al. 2015).

6.6 Concluding remarks and future directions

This study marks an important step forward for utilizing wild relative's especially tertiary gene pool of wheat. Here, It has been successfully demonstrated that production of wheat-alien species chromosome translocation lines by induction of homeologous pairing and recombination is the best approach to transfer alien genes into wheat because these lines contain the gene of interest in a translocated fragment of alien chromatin. They not only contributed minimal amounts of alien chromatin, but they are genetically more stable than amphiploids and addition lines.

Chapter 3 of this work explains the identification and characterization of the small terminal; intercalary and whole arm wheat-*Th. bessarabicum* translocation lines. All six translocation lines were characterized by *in situ* hybridization. The entire *Th. bessarabicum* origin chromosomes involved in translocation were identified. The sizes *of Th. bessarabicum* chromatin segments were also defined. All the lines were disomic (2*n*=42) stable and with good morphological characters.

Chapter 4 describes the effective use of molecular markers to define the wheat alien breakpoint. More markers and whole genome sequence information about *Th. bessarabicum* will be needed to develop more markers to characterize *Th. bessarabicum* origin chromosome segment. The similarity of repetitive sequences within the D genome to those of *Thinopyrum* revealed by the genomic *in situ* hybridization also emphasizes that we need to have stronger data to show the relationships and phylogeny of the various *Aegilops*, *Triticum*, *Thinopyrum* and other genera both with respect to sequence synteny and to karyotype rearrangements. These data will help enable rational

decisions about making and exploiting hybrids. In the work reported here, no attempt was made to identify FHB and other disease resistance genes in *Th. bessarabicum*; more attempts will be needed. Translocation lines used in this study can further be backcrossed again to reduce the size of alien chromatin. The wheat-*Th. bessarabicum* introgression lines used in this study carry genes for salt tolerance and hence will be very useful for future breeding. Multi-location trials are needed and also different wheat backgrounds to Prinia. In summary, all the plant material characterized in this thesis will serve as potential donor material for wheat breeding.

The world faces a potential crisis in terms of future food security. We need to produce and supply enough food, using less land, water, and other natural resources, which will fulfill the food demand of the growing global population. The research work presented here highlights some important wheat breeding efforts, which are undergoing and needed. As wheat fulfills 45% of global population food demand, more research in the field is necessary. With a world undergoing wars and conflicts, we need to focus our attentions towards a more common goal of peace that comes with agriculture together.

As Norman Borlaug rightly said:

"You cannot build a peaceful world on empty stomachs and human misery."

"Without food, man can live at most but a few weeks; without it, all together components of social justice are meaningless."

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Appendix 1: List of SSR markers used in this study

Marker name	Chro-moso-mal location	Primer sequence	Tm^{0}C	Prod uct size (bp)	Polymorphism
Xgwm251	4BL	F: CAACTGGTTGCTACACAAGCA R: GGGATGTCTGTTCCATCTTAG	63^{0}C	110	Not Polymorphic
Xgwm538	4BL	F: GCATTTCGGGTGAACCC R: GTTGCATGTATACGTTAAGCGG	63^{0}C	168	Polymorphic in Thinopyrum
Xgwm375	4BL	F: ATTGGCGACTCTAGCATATACG R: GGGATGTCTGTTCCATCTTAGC	63^{0}C	-	Not Polymorphic
Xgwm149	4BL	F: CATTGTTTTCTGCCTCTAGCC R: CTAGCATCGAACCTGAACAAG	61^{0}C	161	Polymorphic in Thinopyrum
Xgwm165	4BL	F: TGCAGTGGTCAGATGTTTCC R: CTTTTCTTTCAGATTGCGCC	63^{0}C	257	Polymorphic
Xcfd54	4BL	F: TCGTTCCAAAATGCATGAAA R: AAGGGCCAGAAATCTGTGTG	61^{0}C	200	Polymorphic
Xcfd283	4BL	F: CCCGTGGTCTTGGGTTC R: AGTTTTGCCATCGGCTGTAT	61^{0}C	273	Polymorphic
Xcfa2149	4BL	F: CTTGGAGCTCGGGTAGTAGC R: AAGGCAGCTCAATCGGAGTA	63^{0}C	231	Polymorphic
Xgwm6	4BL	F: CGTATCACCTCCTAGCTAAACTAG R: AGCCTTATCATGACCCTACCTT	63^{0}C	207	Not Polymorphic
Xgwm314	4BL	F: AGGAGCTCCTCTGTGCCAC R: TTCGGGACTCTCTTCCCTG	63^{0}C	182	Polymorphic
Xwmc511	4BL	F: CGCACTCGCATGATTTTCCT R: ATGCCCGGAAACGAGACTGT	61^{0}C	202	Not Polymorphic
Xwmc546	4BL	F: CGGCTAAAATCGTACACTACACA R: CTCACTTGCACGATTTCCCTAT	61^{0}C	151	Not polymorphic
Xwmc710	4BL	F: GTAAGAAGGCAGCACGTATGAA R: GTAAGAAGGCAGCACGTATGAA	61^{0}C	126	Polymorphic in Chinese spring
Xgwm120	2BL	F: GATCCACCTTCCTCTCTCTC R: GATTATACTGGTGCCGAAAC	64^{0}C	162	Polymorphic
XbarcM139	2BL	F: AGAAGCTCCCCTAAACTG AG R: CGACGCTGATGAATGAAT	64^{0}C	-	Polymorphic
Xbarc167	2BL	F: AAAGGCCCATCAACATGCAAGTACC R: CGCAGTATTCTTAGTCCCTCAT	64^{0}C	-	Polymorphic
Xwmc602	2BL	F: TACTCCGCTTTGATATCCGTCC R: GTTTGTTGTTGCCATCACATTC	61^{0}C	168	Polymorphic
Xwmc356	2BL	F: GCCGTTGCCCAATGTAGAAG R: CCAGAGAAACTCGCCGTGTC	61^{0}C	242	Polymorphic
Xwmc317	2BL	F: TGCTAGCAATGCTCCGGGTAAC R: TCACGAAACCTTTTCCTCCTCC	61^{0}C	139	Polymorphic
Xwmc175	2BL	F: GCTCAGTCAAACCGCTACTTCT R: CACTACTCCAATCTATCGCCGT	61^{0}C	253	Polymorphic
Xgwm299	3BL	F: ACTACTTAGGCCTCCCGCC R: TGACCCACTTGCAATTCATC	61^{0}C	206	Polymorphic

Xgwm181	3BL	F: TCATTGGTAATGAGGAGAGA R: GAACCATTCATGTGCATGTC	61^0C	150	Polymorphic
Xgwm547	3BL	F: GTTGTCCCTATGAGAAGGAACG R: TTCTGCTGCTGTTTTCATTTAC	61^0C	171	Polymorphic
Xwmc274	3BL	F: AAGCAAGCAGCAAAACTATCAA R: GAATGAATGAATGAATCGAGGC	61^0C	139	Polymorphic
Xgwm291	3BL	F: CATCCCTACGCCACTCTGC R: AATGGTATCTATTCCGACCCG	61^0C	222	Polymorphic
Xwmc727	3BL	F: CATAATCAGGACAGCCGCAC R: TAGTGGCCTGATGTATCTAGTTGG	61^0C	138	Polymorphic
Xwmc632	3BL	F: GTTTGATTGGTCGTTCCTGGTC R: AACAGCGAATGGAGGGCTTTAG	61^0C	180	Polymorphic
Xcfa2170	3BL	F: TGGCAAGTAACATGAACGGA R: ATGTCATTCATGTTGCCCCT	63^0C	199	Polymorphic
Xwmc152	6BL	F: CTATTGGCAATCTACCAAACTG R: TCTCTTCTTGCCACATATTCGT	63^0C	251	Not polymorphic
Xgwm626	6BL	R: GATCTAAAATGTTATTTTCTCTC F: TGACTATCAGCTAAACGTGT	61^0C	101	Polymorphic
Xgwm219	6BL	F: GATGAGCGACACCTAGCCTC R: GGGGTCCGAGTCCACAAC	61^0C	184	Polymorphic
Xgdm147	6BL	F: CAAACAAGGTGGGTTCACTG R: TTTTTGAGTTCAACGGAGAC	61^0C	-	Polymorphic
Xbarc178	6BL	F: GCGTATTAGCAAAACAGAAGTGAG R: GCGACTAGTACGAACACCACAAAA	61^0C	-	Polymorphic
Xbarc134	6BL	F: CCGTGCTGCAAATGAACAC R: AGTTGCCGGTTCCCATTGTCA	61^0C	-	Polymorphic
Xwmc726	6BL	F: GCAAAGAACCGTGCCCTGAC R: CGGGGTGGCCCGAGA	61^0C	179	Polymorphic
Xbarc79	6BL	F:GCGTTGGAAAGGAGGTAATGTTAGATAG R:TCGTGGGTTACAAGTTTGGGAGGTCA	61^0C	-	Polymorphic
Xgwm611	7BL	F: CATGGAAACACCTACCGAAA R: CGTGCAAATCATGTGGTAGG	61^0C	166	Polymorphic
Xgpw1113	7BL	F: CTTCAAGCACCCGCATAAAT R: GGTGTTCCCTGTGACCTCAT	60^0C	245	Polymorphic
Xgpw1045	7BL	F: TTCTCTCGTTTCTTCGGTGG R: CACAATCTGGACGATACCCC	60^0C	231	Polymorphic
Xgpw1144	7BL	F: CTCGAGCGACTAACCCTGTC R: GTGCCGAACTGACCTTGATT	61^0C	247	Polymorphic
Xgwm577	7BL	F: ATGGCATAATTTGGTGAAATTG R: TGTTTCAAGCCCAACTTCTATT	61^0C	164	Polymorphic
Xwmc70	7BL	F: GGGGAGCACCCTCTATTGTCTA R: TAATGCTCCCAGGAGAGAGTCG	61^0C	213	Polymorphic
Xgwm190	5DS	F: GTGCTTGCTGAGCTATGAGTC R: GTGCCACGTGGTACCTTTG	63^0C	201	Polymorphic
Xgpw326	5DS	F: TTTTTGTCCGTTCACCATCA R: ACTCATCTTTCTCTTGCACACC	61^0C	147	Polymorphic
Xcfd165	5DS	TTTCCTTGGATCCACTCACC GAAACAACCCAGGGACAAGA	61^0C	250	Polymorphic

Xcfd18	5DS	F: CATCCAACAGCACCAAGAGA R: GCTACTACTATTTCATTGCGACCA	61^0C	169	Polymorphic
Xcfd189	5DS	F: GCTAAAGCCACATAGGACGG R: GCACAAGATTTTGCAAGGCT	63^0C	280	Polymorphic
Xcfd67	5DS	F: GCGGACAAATTGAGCCTTAG R: TGTGCGTGTGTGTGTGTTTT	63^0C	188	Polymorphic
Xbarc130	5DS	F: CGGCTAGTAGTTGGAGTGTTGG R: ACCGCCTCTAGTTATTGCTCTC	63^0C	-	Polymorphic
Xcfd78	5DS	R: ATGAAATCCTTGCCCTCAGA F: TGAGATCATCGCCAATCAGA	63^0C	182	Polymorphic
Xcfd102	5DS	R: TTGTGGAAGGGTTTGATGAAG F: TGCAGGACCAAACATAGCTG	61^0C	299	Polymorphic
Xbarc205	5DS	R: GCGACAGTTGTAGCGGCAGTAGC F: GAGCGTAGTAGAAGCAGAAGGAG	61^0C	-	Polymorphic
Xcfd81	5DS	F: TATCCCCAATCCCCTCTTTC R: GTCAATTGTGGCTTGTCCCT	63^0C	283	Polymorphic
Xwmc539	5DS	F: GCAAGTAGGACCTTACAGTTCT R: GTTATAACCTTTGTCCCTTCAC	61^0C	199	Polymorphic
Xcfd8	5DS	F: ACCACCGTCATGTCACTGAG R: GTGAAGACGACAAGACGCAA	61^0C	162	Polymorphic
Xgwm205	5DS	F: CGACCCGGTTCACTTCAG R: AGTCGCCGTTGTATAGTGCC	63^0C	158	Polymorphic
Xcfd74	5DS	F: TCAAAACCACACCAGGCATA R: AAGTGGTGGGGAGTGTGTGT	63^0C	338	Polymorphic
Xcdf266	5DS	F: GAAAACAAAACCCATTTGCG R: AAGCTTCAGTGCCTTTGGAA	61^0C	192	Polymorphic
Xgwm136	1AS	GACAGCACCTTGCCCTTTG CATCGGCAACATGCTCATC	63^0C	278	Polymorphic
Xgwm33	1AS	F: GGAGTCACACTTGTTTGTGCA R: CACTGCACACCTAACTACCTGC	63^0C	116	Polymorphic
Xwmc24	1AS	F: GTGAGCAATTTTGATTATACTG R: TACCCTGATGCTGTAATATGTG	63^0C	152	Polymorphic
Xbarc28	1AS	F: CTCCCCGGCTAGTGACCACA R: GCGGCATCTTTCATTAACGAGCTAGT	63^0C		Polymorphic
Xgpw2172	5AL	F: TGACGGGTCACACATCAAAT R: CGAGAAAGAGTAGGGCATGG	63^0C	294	Polymorphic
Xgwm126	5AL	F: CACACGCTCCACCATGAC R: GTTGAGTTGATGCGGGAGG	63^0C	196	Polymorphic
Xgwm595	5AL	F: GCATAGCATCGCATATGCAT R: GCCACGCTTGGACAAGATAT	63^0C	146	Polymorphic
Xgwm179	5AL	F: AAGTTGAGTTGATGCGGGAG R: CCATGACCAGCATCCACTC	63^0C	181	Polymorphic
Xwmc110	5AL	F: GCAGATGAGTTGAGTTGGATTG R: GTACTTGGAAACTGTGTTTGGG	61^0C	170	Polymorphic
Xwmc577	5AL	F: CTGTCCGACTCCCCAGATG R: CCCTGTCAGAGGCTGGTTG	61^0C	124	Non polymorphic
Xwmc524	5AL	F: TAGTCCACCGGACGGAAAGTAT R: GTACCACCGATTGATGCTTGAG	61^0C	198	Polymorphic

Appendix 2: List of standard buffers and solutions used in this study

CTAB buffer (pH 7.5-8.0)	2% (w/v) cetyltrimethylammonium bromide, 100mM Tris-HCL, 1.4 M NaCl, 20mM EDTA
DNA wash buffer	76% ethanol, 10mM ammonium acetate.
10x TE buffer (pH 8.0)	100mM Tris (tris – hydroxymethylamino – methane) – HCL, 10mM EDTA (ethylene diamine – tetra- acetic acid).
6x Gel loading buffer	0.25% Bromophenol blue, 0.25% xylene cyanol FF, 60% Glycerol. Stored at 4ºC.
50x TAE (pH 8.0)	242g of Tris- base, 57.1ml of glacial acetic acid,100 ml of 0.5M EDTA.Final volume 1000ml with sterile distilled water.
Ethidium Bromide (10 mg/ml)	1 g ethidium bromide, 100ml of sterile distilled water. Store at 4ºC.
20x SSC (Saline sodium citrate, pH 7.0)	0.3 M NaCl, 0.03M sodium citrate.
10x PBS (phosphate buffer saline,pH 7.4)	1.3M NaCl, 70mM Na2HPO4, 30mM NaH2PO4
Detection buffer (FISH)	4X SSC, 0.2% (v/v) tween 20
10x kpbs (potassium phosphate buffered saline pH 7.4)	1.28 M NaCl, 20mM KCL, 80mM Na2HPO4, 20mM NaH2PO4.
10X Enzyme buffer (pH 4.6)	40mM citric acid, 60mM tri-sodium citrate. Stored at 4ºC.
1x Enzyme solution	3% (w/v) pectinase (Sigma), 1.8% (w/v) cellulase (Calbiochem), 0.2% (w/v) cellulase (Onozuka RS) in 1X enzyme buffer. Stored at -20ºC.
4% Paraformaldehyde (pH 7.0)	4% Paraformaldehyde (Agar Scientific) dissolved in distilled water. Final volume 100 ml.

McIlvaines buffer (pH 7.0)	0.1 M Citric acid, 0.2M di-sodium hydrogen phosphate.
Blocking DNA for *in situ* hybridization.	Autoclaved at 110ºc for 3 mins
100 µg/ml DAPI	5g of DAPI (4',6-diamidino-2-phenylindole) dissolved in Sigma water. Final volume 50ml. Stored at -20ºC.
Buffer 1 (probe detection pH 7.5)	100mM Tris- HCL 15Mm NaCl
Buffer 2 (probe detection)	0.5%(w/v) Blocking reagent (Roche Diagnostics) in buffer 1.
Buffer 3 (probe detection pH 9.5)	100mM Tris- HCL, 100mM NaCl, 50mM MgCl2
Salmon sperm DNA	1mg/ml of sheared salmon sperm DNA.

			(Days)	14-Aug	14-Aug	21-Aug	21-Aug	28-Aug	28-Aug				
Line	Pedigree	Translocation	Heading (wt)	***Septoria tritici***						Estimate1	Estimate2	Estimate3	AUDPC
Prinia	Prinia	Parent	60	3	1	6	4	8	4	3.7037	29.6296	39.5061	358.6419
Line1160	CS/TH.BESS//CSph/3/4*PRINIA	T4BS.4BL-4JL	67	5	3	8	6	9	9	18.5185	59.2592	100	829.6296
Line1164	CS/TH.BESS//CSph/3/4*PRINIA	T6BS.6BL-6JL	73	5	2	9	5	9	9	12.3456	55.5555	100	782.0987
Line1172	CS/TH.BESS//CSph/3/4*PRINIA	T5DL.5DS-5JS	67	5	2	8	5	8	5	12.3456	49.3827	49.3827	561.7283
Line1176	CS/TH.BESS//CSph/3/3*PRINIA	T2BS.2BL-2JL	67	6	4	9	6	9	9	29.6296	66.6666	100	920.3703
Line1180	CS/TH.BESS//CSph/3/4*PRINIA	T1AL.1JS	71	5	3	8	4	9	5	18.5185	39.5061	55.5555	535.8024

Appendix 3: Scoring for *Septoria tritici*

				Total of spikes										Total number of infected spikes													
	Translocation	Date of Inoculation.	Height	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	**Ave Tot**	**Ave Dam**	**Damage (%) Type I**	**Incidence (%)**	**FHB index**
PRINIA	Parent	5/8/2013	90	17	19	16	17	18	18	16	17	17	19	0	1	1	1	1	0	0	0	1	0	17.4	0.50	2.87	50.00	1.44
Line1160	T4BS.4BL-4JL	5/8/2013	80	20	18	18	19	19	19	19	18	16	17	1	3	3	0	3	2	4	2	0	4	18.3	2.20	12.02	80.00	9.62
Line1164	T6BS.6BL-6J	5/8/2013	65	16	17	17	17	18	16	17	18	20	20	0	0	0	0	0	0	0	0	0	0	17.6	0.00	0.00	0.00	0.00
Line1172	T5DL.5DS-5JS	5/8/2013	60	15	14	14	15	15	17	14	16	15	15	3	3	3	5	1	2	2	2	2	2	15.0	2.50	16.67	100.00	16.67
Line1176	T2BS.2BL-2JL	31/7/2013	100	17	17	19	19	19	19	19	19	18	19	4	6	7	6	3	1	2	5	1	4	18.5	3.90	21.08	100.00	21.08
Line1180	1AL.1JS	7/8/2013	105	19	19	19	13	19	21	19	19	19	20	0	1	3	0	0	1	2	2	1	0	18.7	1.00	5.35	60.00	3.21
Disomic Addition	1J	9/8/2013	60	15	19	17	17	19	19	19	18	19	18	0	3	2	3	0	1	1	3	2	0	18.0	1.50	8.33	70.00	5.83
Disomic Addition	2J	31/7/2013	75	16	17	15	16	18	17	17	16	15	17	3	3	6	1	0	6	0	2	3	2	16.4	2.60	15.85	80.00	12.68
Disomic Addition	3J	29/7/2013	100	25	24	23	23	21	24	23	22	21	21	0	0	0	0	0	0	0	0	0	0	22.7	0.00	0.00	0.00	0.00

Appendix 4: Scoring for FHB (Fusarium Head Blight)