The use of pentaploid crosses for durum wheat improvement to Septoria tritici blotch disease resistance and D-genome introgression into durum wheat

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Abstract

Pentaploid hybrids derived from crosses between bread wheat (*Triticum aestivum*) and durum wheat (*Triticum turgidum*) have the ability to improve the genetic background of either parent by transferring traits of interest.

Septoria tritici Blotch (STB) disease is a major wheat problem of durum wheat in the Mediterranean area, especially in Tunisia. Using pentaploid crosses, resistant genes were transferred from three hexaploid wheats, into two susceptible durum wheat genotypes. *Stb7/12*, *Stb6* and *Stb17* were found to be ineffective when transferred into durum wheat. However, the segregation of the populations developed for STB disease resistance in field conditions indicated the presence of resistance gene(s) in hexaploid wheat that can be used for durum wheat improvement. Genotyping by sequence analysis of four selected pentaploid populations enabled the quantification of the genetic variability inherited from the bread wheat parent. Results showed that more genetic variability was captured when using the one durum wheat compared to the other, suggesting the importance of the parental choice in pentaploid crosses.

The D-genome progenitor of wheat, *Ae. tauschii* has been used as a source of resistance/tolerance genes to biotic and abiotic stresses, including resistance genes to many fungal diseases of bread wheat. However, only a very few studies have focused on the use of *Ae. tauschii* for durum wheat improvement. With the aim to develop durum wheat/*Ae*. *tauschii* introgression lines, the Langdon 5D (5B) disomic substitution line which lacks the *Ph1* gene, was crossed to *Ae. tauschii*. The F_1 amphihaploid was then crossed and backcrossed to the durum wheat "Om Rabiaa 5". Using a cytogenetic screening approach via GISH and FISH, in combination with D-genome specific SSR markers, tetraploid D-genome introgression lines were identified and characterised.

The introgression of D-genome translocations, present in the genetic background of hexaploid/*Am. muticum* introgression lines, was shown to be an efficient crossing strategy to make use of these D-segments. Translocation were traced via mc-GISH and introgression lines were characterised via mc-FISH. The presence of *Am. muticum* segment in some of the introgression lines promoted the occurrence of new genomic translocations in the backcross generations to the durum parent. Homozygous D-genome introgressions plus simultaneous homozygous D- and T-genome introgressions were distinguished in a tetraploid background.

The D-genome and/or T-genome introgression lines produced will be screened for STB disease resistance as well as other traits of interest.

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Contents

List of Figures

List of tables

List of Abbreviations

µl: microlitre
A: Adult
Ae.: *Aegilops*
AFLP: Amplified fragment length polymorphism
Al: Aluminium
Am.: *Amblyopyrum*
AUDPC: Area under the disease progress curve
B: Borne
BFC: Brakeage-Fusion-Bridge
bp: baize pair
CIMMYT: International Centre of Maize and Wheat improvement
cM: Centimorgan
CS: Chinese spring
Cv.: cultivar
DAPI: 4',6-diamidino-2-phenylindole
DArT: Diversity Array Technology
DNA: Deoxyribonucleic acid
dpi: day post inoculation
EDTA: Ethylene-diamine-tetraacetic acid
FAO: Food and agriculture organization
FCR: Fusarium crown rot
FHB: Fusarium head blight
FISH: Fluorescent *in situ* hybridization
g: gramme
GBS: Genotyping by sequencing
GFG: Gene-for-gene
GISH: Genome *in situ* hybridization
GWAS: Genome-wide association study
h: hour
ha: hectares
HCN: Hydrogen cyanide
IWGSC: International Wheat Genome Sequencing Consortium
KR: Karim
LA: Long arm
LND-DS: Langdon D-genome -Disomic Substitution
MAS: Marker Assisted Selection
MATI: mating type idiomorph
mc: Multi-colour
Mins: minutes
ml: millilitre
mM: millimolar
MR: Moderate resistant

MS: moderately susceptible
N: Nitrogen
NGS: Next generation sequencing
OR: Om Rabiaa 5
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
***Ph I*:** Pairing homoeologous inhibitor
***Ph1*:** Pairing homoeologous 1
PM: Powdery mildew
QTL: Quantitative trait loci
R: Resistant
RAPD: Random Amplification of Polymorphic DNA
RFLP: Restriction fragment length polymorphism
RobT: Robertsonian translocation
S: Susceptible
SA: Short arm
sc: single colour
SC: Synaptonemal complex
SDHI: Succinate dehydrogenase inhibitor
SDL: Synthetic derived line
SDS: Septoria disease severity
SHW: Synthetic Hexaploid wheat
SNB: Septoria nodorum blotch
SNP: Single nucleotide polymorphism
SPPP: Septoria precision phenotyping platform
SR: Stem rust
SSC: Saline-sodium citrate
SSR: Single Sequence Repeat
STARP: Semi-thermal asymmetric reverse PCR
STB: Septoria tritici blotch
T.: *Triticum*
TKW: Thousand kernel weight
Tris-HCl: Tris hydrochloride
USDA: United State Department of Agriculture
UV: Ultraviolet
V: volume
WAK: Wall-Associated receptor kinase
YR: Yellow rust
***Z. tritici*:** *Zymoseptoria tritici*
µg: microgram

1 Chapter I. General introduction

1.1 Introduction

Cereals crops are the most important global food; they make up around 50% of the global food production (http://faostat.fao.org). Wheat (*Triticum* spp.) is the second most important staple food crop after rice. Humans have cultivated and consumed wheat since the beginning of civilisation. In fact, wheat is the number one crop consumed per person per year. 67% of the wheat produced worldwide is used for human consumption (FAO, 2016).The highest consumption per person per year is in central Asia (143kg), followed by North Africa (139kg), western Asia (138kg) and Europe (109kg). Wheat is an important source of calories in human diets; it provides one-fifth of the total calories of human's food to more than 4.5 billion people in at least 94 developing countries (Ray *et al.*, 2012).

The most important cultivated wheat species are hexaploid bread wheat (*Triticum aestivum*) and tetraploid durum wheat (*Triticum turgidum* L. var. *durum*), that are different from one another in grain composition and end-use quality. Wheat has been used for the manufacture of numerous products, mainly bread, noodles, pasta and beer. About 90-95% of the wheat produced in the world is bread wheat, which can be hard wheat or soft wheat, depending on grain hardness. Hard grain bread wheat is mainly used as flour for the production of a large variety of leavened and flat breads. Soft bread wheat grain are instead used for the manufacture of a wide variety of other baking products such as biscuits. Durum wheat is the most widely grown of the tetraploid wheats. Due to its unique qualities such as its hardness, high protein content, and gluten strength, it's mainly used for the production of high quality pasta worldwide, along with being the raw material for the production of couscous (North Africa), bulgur (Turkey), various types of bread (Mediterranean and West Asian countries), firik (North Africa and Middle East), among others (Magallanes-Lopez *et al.*, 2017).

Wheat is one of the important species whose cultivation and domestication has been closely associated with the prosperity of agriculture and settled societies. Nowadays, food security is a key challenge facing humanity, given

the exponential population growth and climate change (Schmidhuber and Tubiello, 2007; Beddington *et al.*, 2012). Wheat production is highly affected by various abiotic and biotic stresses that are key yield limiting factors. Increasing production without agricultural expansion remains at the core of proposed solutions to ensure food security while minimizing environmental impact. Hence, breeding for high yielding, good quality, disease resistant, and adapted to a broader range of environmental conditions cultivars has become an urgent need to meet the food demand.

1.2 Wheat origin and evolution

Historically, different species of *Triticum* genus have been used under the wheat denomination. These species are distributed in three ploidy levels: diploid (2n=2x=14), tetraploid (2n=4x=28) and hexaploid (2n=6x=42). The allopolyploids arose from interspecific hybridisation events as a consequence of a cross with species from the genus *Aegilops* followed by spontaneous chromosome doubling (Huang *et al.*, 2002) (**Figure 1-1**). Wheat origin and domestication are placed in the Near East, in the zone known as the Fertile Crescent (Zohary and Hopf, 1993). Here, two wild species were domesticated by the primary agricultural societies. One diploid, *T. monococcum ssp. aegilopoides* (Link) Thell.; genome (2n=2x=14, A^mA^m) and the other tetraploid *T. turgidum ssp. dicoccoides* (Korn. ex Asch. & Graebner) Thell.; (2n=4x=28, A^uA^uBB). This led to the cultivation of two wheat species: einkorn wheat (*T. monococcum*) and emmer wheat (*T. turgidum spp. Dicoccum*).

Emmer wheat, is the primary cultivated tetraploid wheat and is considered to have risen 500,000 years ago, from a cross between the wild ancestors of the A- and the B-genomes (Huang *et al.*, 2002). Studies confirmed that the origin of the A-genome was *T. urartu* Thum ex. Gandil (2n=2x=14, A^uA^u) (Feldman and Levy, 2005). However, the origin of the B-genome remains controversial. It has been associated with *Ae. speltoides* (2n=2x=14, SS), but has also been related to different *Aegilops* species from the Sitopsis section of the Triticeae (Tsunewaki and Ogihara, 1983; Feldman, 2001). The expansion and the cultivation of this tetraploid species led to a spontaneous cross of this crop as the female parent with one goat grass of the Caspian Sea zone *Ae. tauschii* Coss. (2n=2= 14, DD), creating a new species *T. aestivum ssp. spelta* (L.) Thell.;

(2n=6x=42, A^uA^u BB DD) from which derived the bread or common wheat (*T. aestivum L. ssp. aestivum*) approximately 8,000 years ago (Kihara, 1944; McFadden and Sears, 1944). Bread wheat became one of the most widely grown crops due to its high yields and nutritional and processing qualities (Shewry and Hey, 2015).

Archaeological evidence shows that the earliest domesticated wheats from the Fertile Crescent spread to the west of the Mediterranean Basin, via Turkey (8,500 B.P.), and the Balkan Peninsula, Greece and Italy (8,000 B.P.), and from there to North Africa reaching the Iberian Peninsula (7,000 B.P.) (Feldman, 2001; MacKey, 2005). This process of migration and both natural and human selection resulted in the establishment of local landraces specifically adapted to a diversity of agro-ecological zones.

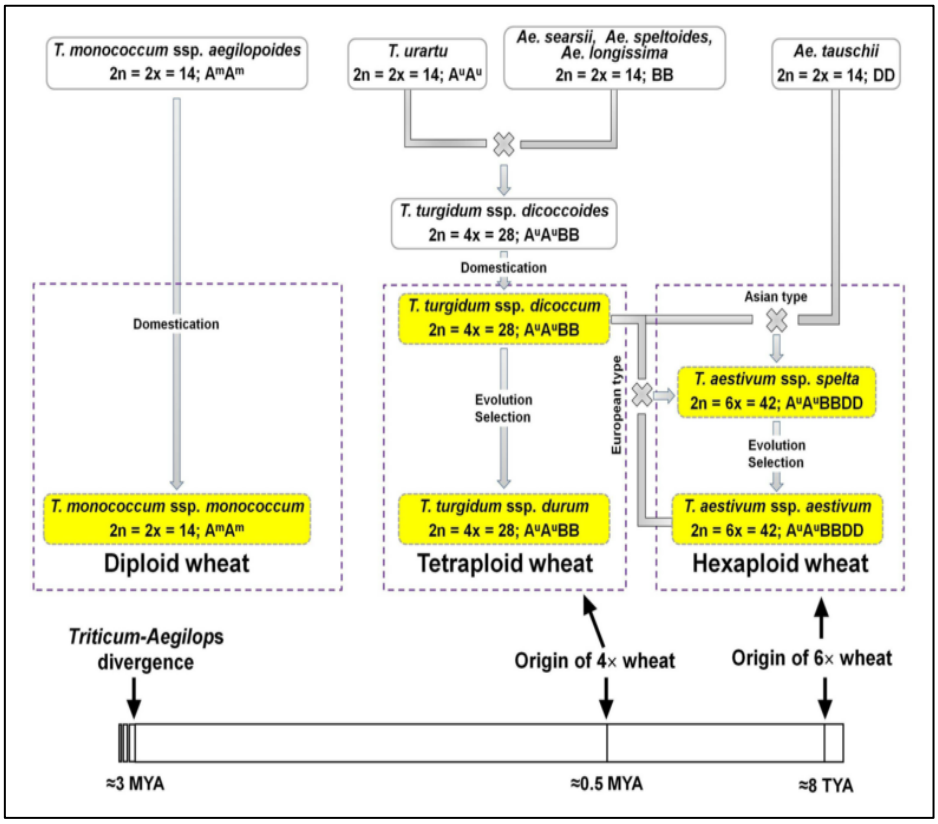

Figure 1-1. Diagrammatic representation of the species included in the wheat complex. The species in yellow are or have been cultivated at some point during history. The timeline shows the main events in the wheat evolution (Alvarez and Guzman, 2017).

1.3 Wheat production

Landraces were widely grown until the early 1970s, when they were rapidly displaced by homogeneous and more productive semi-dwarf cultivars. In fact,

since the 1960s, increases in wheat production have been achieved after a wide-scale adoption of the green revolution varieties (Evenson and Gollin, 2003). This dramatic productivity increase worldwide was essential in raising wheat production sufficiently to alleviate the effect of rapid demographic growth. This was aided by the fact that wheat can be cultivated at diverse latitudes and altitudes under irrigated, severe drought, and wet conditions (Singh *et al*., 2016). Wheat domestication, thus, occurred under variable climates including tropical, subtropical and temperate. As a result, three types of wheat evolved; known as winter, spring, and facultative wheats. They differ in temperature response due to the presence and absence of dominant vernalisation genes (Sun *et al.*, 2009).

The global production of bread wheat in 2016 was estimated at about 749 million tonnes, grown on 220 million hectares throughout the world (FAO, 2016) and with an average yield of 3.4 t/ha (FAO, 2016). The world's major bread wheat-producing areas are in northern China, northern India, northern USA and adjoining areas in Canada, northern and central Europe, western Russia, southern Australia, southern Latin America and South Africa. Unlike bread wheat, which is basically cultivated worldwide with the exception of the tropical areas, durum wheat is grown on about 8% of the world's wheat area (FAO, 2016), that is mainly in West Asia, North, and East Africa, the North American Great Plains, India, Eastern and Mediterranean Europe (International Wheat Council, 1991). Durum wheat is the 10th most important crop worldwide with a total annual production of 37 million tons (Ranieri, 2015; Taylor and Koo, 2015). Europe and North Africa are the largest importers of durum wheat (Bonjean *et al.*, 2016). Despite the relatively small growing area and the lower annual production of durum wheat compared to bread wheat, durum wheat remains a major crop in the Mediterranean basin where about 75% of the world's durum wheat is produced (Li *et al.*, 2013; Kabbaj *et al.*, 2017). Indeed, the Mediterranean area is the largest consumer of durum wheat products.

1.4 Food security

Wheat is an important crop and represents an essential component of the global food security. As the potential of increasing arable land is limited, the availability of water is decreasing and the population is increasing. Demand for wheat is projected to rise at a rate of 1.6% annually until 2050 (Singh *et al*.,

2016). As a result, average global wheat yields per hectare will need to increase to approximately 5 tonnes per ha from the current yield levels of 3 tonnes (Buerstmayr *et al*., 2012). However, the annual growth rate of global wheat production is below one percent, which eventually cannot meet the global market requirements during the next four decades (Fischer *et al*., 2009; Fischer and Edmeades, 2010). Food production may need to increase by 50% as predicted over the next 25 years when the global population may likely reach 9 billion people (World Bank, 2008). Food security depends not only on gross production of staples, but also on the ability to provide an income for farmers in developing countries, a diverse and balanced food basket, and the socio-economic factors that determine whether poor people, particularly women, are able to purchase, store, prepare and consume sufficient food (Velu and Singh, 2013). Breeding cereals with improved yield and quality performance has become an urgent and relevant need to ensure food security.

1.5 Wheat production constraints

1.5.1 Abiotic stresses

Wheat production needs to breed cultivars that are resistant/tolerant to a wide range of abiotic stresses. Damaging abiotic stresses that affect yield are mainly drought, salinity, heat and mineral toxicities. Heat and drought are the major abiotic stresses directly affected by climate change. Disruption in normal climate cycles can highly affect wheat production. Water deficit, caused by drought, results in changes in morphology, water status, gas exchange and chlorophyll content which in turn are directly connected with the onset of protective mechanisms in the plant (Blum and Ebercon, 1981; Mansfield and Davies, 1981; Jackson *et al*., 1996). Drought is significant in marginal and rain-fed areas of wheat cultivation, where timing and intensity of drought stress are variable due to continuously changing climatic factors (Passioura *et al*., 1993; Bohnert *et al*., 1995; Blum, 1998; Chaves *et al*., 2002; Tan *et al*., 2006). For instance, spring wheat, a widely grown dryland crop, is the main food source in semiarid regions, and climate change can directly induce a considerable decrease in yield in these regions (Dong *et al.,* 2018). High temperatures during crop growth and grain filling stages are also of major concern to wheat production as they can negatively influence the grain yield and quality (Branlard

et al., 2015). Liu *et al.* (2016) predicted that wheat yields could decline by 4.1% to 6.4% for each global increase of 1°C due to climate change. Another study carried out by the World Bank predicted that a 2°C rise in world average temperature might lead to extreme heat conditions in South Asia with estimated losses of 6–20% per degree rise in temperature (Lobell *et al*., 2008).

Salinity is another major abiotic stress which affects crop productivity. The problem becomes more acute due to irrigation with saline water and results in uncultivable saline/sodic soils. Moreover, acid soils are known to severely limit plant growth on about 1.6 billion hectares worldwide, which are estimated to be approximately 30–40% of arable lands in the world (Lilienfein *et al*., 2003). Aluminium toxicity is a major growth-limiting factor in acid soil. As the pH falls below 5.0, Al^{3+} becomes the dominant form of aluminium in the soil. This inhibits the elongation and the division of plant root tips, and subsequently reduces nutrient and water uptake. The same effect can be caused by boron (B) toxicity typical in alkaline and saline soils. Boron toxicity significantly reduces wheat yields through affecting the plant growth (Kalayci *et al*., 1998; Sharma *et al*., 2005). In soil, B exists mainly as boric acid (H_3BO_3). The insufficient leaching of H_3BO_3 under low rainfall conditions may lead to the accumulation of boron which becomes toxic to plants (Nable *et al*., 1992). On the contrary, under high rainfall conditions, over leaching of B from the soil can lead to B deficiency (Shorrocks, 1997). The next 40 years will have to deal with the potentially profound damage to farming from climate change, which in some parts of the world could reduce yields by one third (Velu and Singh, 2013).

1.5.2 Biotic stresses

Biotic stresses, caused by many diseases and insect pests, have a major role in reducing wheat yield and quality. Although bread wheat and durum are vulnerable to attack by a large number of diseases and pests, less than 20 diseases and about five insects or mite pests are of major significance (McIntosh, 1998). Some of these have a global distribution, occurring in most wheat-growing areas, whereas others are restricted to certain geographic regions or climatic zones, and where they can be extremely damaging to crop production. Severe disease infections can reach epidemic levels and cause serious yield losses. Fungal diseases such as rusts (stripe rust, leaf rust and stem

rust), fusarium head blight, powdery mildew, karnal bunt, tan spot, take all, smuts, septoria nodorum blotch (SNB), and septoria tritici bloch (STB) are of economic importance and represent major constraints to wheat production in different parts of the world. The impact and the distribution of these diseases depends not only on the method and the effectiveness of their transmission from one season to another and to different geographical places, but also on the virulence shifts of their pathotypes/races, their population dynamics, and subsequently the emergence and adaptation of new virulent strains. Among the major fungal pathogens, rusts are the most important wheat diseases.

While breeders are in a continuous search for new resistance genes combining them together in one resistant cultivar, new virulent pathotypes continue to evolve which present a constant threat such as the occurrence of the Ug99 stem rust strain. Recently, STB caused by *Zymoseptoria tritici* is an important hemibiotrophic disease of wheat worldwide (Eyal *et al*., 1987; Leath *et al*., 1993; Dean *et al*., 2012). STB disease recently became a significant target in wheat breeding, much more than other well-known diseases such as the rusts and powdery mildew, as it is now a potentially damaging disease throughout the temperate regions on both bread and durum wheat (HGCA, 2012; O'Driscoll *et al*., 2014; Gurr and Fones, 2015). Under favourable climate conditions (notably a Mediterranean climate), STB disease can cause significant yield losses of up to 50%, as it becomes more prevalent and often reaches epidemic levels (Eyal *et al.,* 1985). Thus one of the major challenges in plant breeding is the development of cultivars with multiple resistances to several diseases.

1.6 Wheat Breeding

Modern breeding and monoculture cropping have greatly improved yield and quality, but might have resulted in a reduction of genetic variation potentially making crops more vulnerable to disease and climate change (Winfield *et al.*, 2018). Hence, novel sources of resistance and enhanced genetic variability are required to allow breeders to face the challenge of increasing wheat yields. The mixing of alleles through recombination gives rise to genetic variation, which is fundamental for selection and by which progress in plant breeding can be made. The aim of plant breeders is to reassemble desirable inherited traits, such as simple inherited agronomic characteristics like height and flowering time,

alongside resistance to a spectrum of prevalent diseases, improved quality parameters determined by end use, and high yield based on multi-location trials (Braun *et al.,* 2010). In order to increase and exploit new genetic diversity in wheat, breeders adopt several breeding strategies. Desirable traits can be found in accessions from germplasm banks, where the search usually includes landraces, wild relatives or related genera.

1.6.1 Exploitation of landraces

One way to recover some of the loss in genetic variability is through exploitation of genetic resources available in gene banks (Brown *et al.,* 1989; Vikram *et al.,* 2016) such as the old landrace accessions that may hold novel variability not present in modern varieties (Lopes *et al.,* 2015; Riaz *et al.,* 2016; Vikram *et al.,* 2016). Landraces could be a potential source of resistance to both drought (Reynolds *et al.,* 2007) and diseases (Bansal *et al.,* 2011; 2013; Burt *et al.,* 2014; Toor *et al.,* 2013). They may also harbour valuable variation for traits associated with good agronomic performance and quality. For instance, the pleiotropic adult plant resistant gene *Lr34/Yr18/Pm38* originated from a Chinese landrace (Dakouri *et al.,* 2014) and has been shown to be present at high frequency in Chinese wheat landraces ; 85.1% of 422 landraces (Yang *et al.,* 2008). *Lr34* has been successfully used in CIMMYT wheat breeding programs (Singh, 1993; Singh *et al.,* 2005; Kolmer *et al.,* 2008; Liang *et al.,* 2009; Wu *et al.,* 2015a; b). In a search for resistance to STB disease, a major resistance gene was identified in the Tunisian durum wheat landrace accession "Agili" (Medini *et al.,* 2014). In addition, Emmer wheat, the oldest tetraploid crop in the world (Zohary and Hopf, 1993; Damania, 1998), represents a valuable genetic resource to improve resistance to biotic and abiotic stress in bread wheat and durum wheat (Zaharieva *et al.,* 2010). It has been used in the development of popular bread and durum wheat cultivars. For example, the stem rust resistant bread wheat cultivars 'Hope' and 'H-44' which resulted from a cross between the rust resistant 'Yaroslav emmer' and the bread wheat cultivar 'Marquis' (McFadden, 1930).

1.6.2 The reconstitution of wheat

The reconstitution of wheat from its progenitor species can add new genetic diversity into the wheat gene pool. Tetraploid wheat can be crossed with the

wild diploid D-genome progenitor *Aegilops tauschii*, resulting in synthetic hexaploid wheat (SHW) after chromosome doubling. This approach was widely explored by the International Maize and Wheat Improvement Centre (CIMMYT) and other breeding programs (Mujeeb-Kazi *et al.,* 1996). As the SHWs frequently carry unfavourable alleles and are agronomically poorly adapted, they are typically crossed and then back- or top-crossed to elite bread wheat to produce more agronomically acceptable synthetic derived lines (SDLs). Evaluation of SHWs and SDLs showed extensive new variation in morphological, physiological and agronomic traits (Villareal *et al.,* 1994, 1994b; Ortiz-Monasterio *et al.,* 2007; Dreccer *et al.,* 2008; Rattey *et al.,* 2011), in milling and baking quality traits (Kunert *et al.,* 2007), and in resistance to both biotic (Mujeeb-Kazi *et al.,* 2004; Ogbannaya *et al.,* 2008; Zwart *et al.,* 2010; Mulki *et al.,* 2013) and abiotic stresses (Ogbannaya *et al.,* 2007; De Leon *et al.,* 2011; Ghaffary *et al.,* 2012). Another approach to exploit the D-genome donor, *Ae. tauschii,* for bread wheat improvement is through a direct cross between the two species. Since the D-genome of *Ae. tauschii* is completely homologous and freely recombines with the D-genome of bread wheat (Riley and Chapman, 1960; Thomas and Whelan, 1991), a direct cross with elite bread wheat can be carried out where recombinant chromosomes between the diploid and the hexaploid D-genome are produced.

1.6.3 The use of the wild relatives

In breeding, continual reselection within restricted gene pools is likely to lead to diminishing returns, so it is imperative to introduce new allelic diversity by exploiting non-conventional sources (Reynolds *et al.,* 2011). Wild relatives represent an alternative source to increase the genetic variability of current crops (Friebe *et al.,* 1996; Qi *et al.,* 2007; Feuillet *et al.,* 2008). Wild relatives are a potential source of genetic diversity and a rich source of valuable agronomic traits as well as an important source of disease and insect pest resistance genes (Ceoloni *et al*., 2014; Zhang *et al*., 2006; Warburton *et al*., 2006; Dreisigacker *et al*., 2008; Ortiz *et al*., 2008). The main draw back in using wild relatives is the undesirable traits linked to useful traits known as linkage drag that are not desirable for modern crops. For this purpose, several backcrosses to the modern wheat are required to recover desirable agronomic traits in the newly

synthesised materials. However, only a few genes have been extensively exploited in wheat breeding. Genealogical analysis of accessions in the global *Triticum* gene pool database GRIS4.0 showed that the use of *Aegilops* in wheat breeding began about half a century ago with a gradual increase in the proportion of varieties with *Aegilops* genetic material from 1962 to 2011 (Martynov *et al*., 2015). On the other hand, pedigree analysis of newly released varieties showed that the gene pool of wheat wild relatives was used in only 10% of the crosses (Martynov *et al*., 2015).

The screening of wild relative accessions for several wheat diseases and their adaptation to abiotic stress such as drought and salinity revealed their importance for wheat improvement. Several studies have shown that *Aegilops* species are valuable sources of useful traits, notably as potential sources of resistance genes to many fungal diseases (Soshnikova, 1990). For instance, the diploid species *T. monococcum* and *T. boeoticum* showed high level of resistance to rust diseases and genes of resistance to stem rust and leaf rust from both species have been transferred into wheat (Soshnikova, 1990; Valkoun *et al*., 1989). The wheatgrass species *Thinopyrum ponticum* (Podp.) (2n=10x=70, JJJ^sJ^s) was also shown to carry many valuable genes/QTLs for disease resistance and quality traits (Li and Wang, 2009; Ceoloni *et al*., 2014) such as a QTL for FHB resistance. This QTL has been successfully transferred to durum wheat and conferred a good level of resistance to FHB (Forte *et al*., 2014). Another wheatgrass *Thinopyrum intermedium* (2n=42; EE $E^{st}E^{st}$ StSt) is one of the most valuable sources of highly effective resistance genes in wheat breeding (Salina *et al*., 2015).

Disomic substitution lines of wheat alien species have helped facilitate the detection and localisation of useful genes for several traits in the alien species. For example, these lines were used to study the effect of each wheatgrass chromosome through the phenotyping of the alien chromosome or the deletion of the corresponding wheat chromosome. The substitution of chromosome 6D of bread wheat by the homoeologous chromosome 6Ai#2 of *Thinopyrum intermedium*, conferred a resistance to leaf rust and powdery mildew, and a moderate resistance to stem and yellow rust (Salina *et al*., 2015).

The effect of the alien genes transferred into wheat can be either directly or indirectly related to yield improvement. Genes controlling the number of

kernels per spike and the kernel weight are directly related to yield. As an example, chromosome 6P of the tetraploid wheatgrass *Agropyron cristatum* (L.) Beauv. (2n=4x=28, genome PPPP) when added to, or substituted into hexaploid wheat, induces a superior numbers of florets and kernels per spike (Wu *et al*., 2006). Genes of resistance to insect pests and diseases are considered to be indirectly related to yield. However, they have a great impact on yield through the reduction of crop loss. Several resistance genes to wheat diseases were transferred from wild related species. The introgression of the short arm of chromosome 1R of rye in the form of 1BL/1RS or 1AL/1RS translocations in wheat, represent the most successful example of alien introgression (Trethowan and Mujeeb-Kazi, 2008). This introgression allowed the transfer of useful resistance genes to a variety of diseases that in turn led to a significant yield increase (Trethowan and Mujeeb-Kazi, 2008). The main reason for the limited use of the wild relatives is the complexity of the transfer of genetic material to cultivated species (Monneveux *et al*., 2000). The synthesis of F_1 hybrids between wheat and an alien species is only the first step towards transferring alien genes into wheat. After crossing, chromosomal crossover takes place during meiosis in the F_1 gametes and results in a chromosome with a completely different chemical composition from the two parent chromosomes. This kind of chromosome segment exchange or translocations during meiosis are very rare in wheat/alien crosses. In fact, the presence of genes controlling chromosome pairing in wheat such as the *Ph1* gene (see below) prevents the alien chromosomes from recombining with the wheat ones. For this reason, specific crossing techniques and strategies can be employed for alien introgressions into wheat.

1.7 Meiotic pairing in wheat

1.7.1 *Ph1* locus

In most allopolyploid plants, only homologous chromosomes pair at meiosis. Pairing between homoeologous chromosomes (heterogenetic chromosome pairing) is excluded (Jenczewski and Alix, 2004). In the same way, a genetic restriction of chromosome pairing to only homologous chromosomes was identified in tetraploid and hexaploid wheat. For example, 1A only pairs with 1A but not with either 1B or 1D. Two types of genes in wheat, promoters and

suppressors, affects meiotic chromosome pairing in wheat itself or in hybrids of wheat and related species were identified (Sears, 1976). The *Ph1* (*Pairing homoeologous 1*) gene, located on the long arm of chromosome 5B (5BL), is considered as the main and the most effective suppressor gene of homoeologous pairing, responsible for the meiotic diploid-like behaviour of wheat chromosomes.

In 1977, Sears induced a recessive mutation in the *Ph1* locus, designated as *ph1b* through X-ray irradiation of pollen of the bread wheat cultivar Chinese Spring (CS). This mutation allows homoeologous pairing with bread wheat and a high level of pairing in interspecific and intergeneric hybrids. The *ph1b* mutation (a deletion of a segment of the chromosome) is located near the middle of chromosome arm 5BL, about 1.0 cM (Sears, 1984) to several centiMorgans (cM) (Gill *et al.*, 1993) from the centromere. Another high-pairing mutation, *ph1c*, was induced by X-ray irradiation of the durum cultivar Cappelli (Giorgi, 1978). This mutation is a microscopic deletion in the middle of the 5BL arm that includes the *Ph1* locus (Dvořák *et al.*, 1984; Jampates and Dvorak, 1986). Durum haploids with *ph1c* showed considerable homoeologous pairing (Jauhar *et al.*, 1999).

The *Ph1* locus affects the premeiotic alignment of the homologous and homoeologous chromosomes (Feldman, 1993) and acts by suppressing homoeologous pairing during meiosis (Okamoto, 1957; Sears and Okamoto, 1958; Riley and Chapman, 1958; 1964; Riley and Kempana, 1963). In its absence, extensive homoeologous recombination takes place irrespectively of the length of the homoeologous segment, its location on the centromere-telomere axis, or the homology of both telomeric and centromeric regions (Dubcovsky *et al.*, 1995; Luo *et al.*, 1996). Since the deleted segment of chromosome 5B in the *ph1b* line is about 70 megabase in size, a number of genes are likely to have been deleted (Gill *et al.*, 1993; 1996). Griffiths *et al.* (2006) characterised and localised the *Ph1* locus to a 2.5 MB (megabase) region on chromosome 5B. A gene within this region (corresponding to the wheat gene EST BE498862 and the rice gene Os9g30320), which is expressed during metaphase I, termed *C-Ph1*, has been proposed as the putative *Ph1* gene (Bhullar *et al.*, 2014).

Synapsis is a process early in meiosis by which homologous chromosomes intimately align with each other, forming bivalents held together by a proteinaceous structure named synaptonemal complex (SC). Ultimately, the SC is degraded, so that the bivalents are only held together by chiasmata or crossovers at metaphase I, allowing their correct segregation. The *Ph1* locus has been shown to have a dual effect on synapsis and crossover formation in wheat (Martin *et al.,* 2014; 2017). The effect on synapsis occurs during the telomere bouquet stage, when *Ph1* promotes more efficient homologous synapsis, while the homoeologous pairing starts later during telomere bouquet dispersal (Martin *et al.,* 2017). In wheat lacking *Ph1* locus, homologous synapsis is delayed with respect to the telomere bouquet, with more synapsis occurring after the telomere bouquet stage, when homoeologous synapsis is also possible (Martin *et al.*, 2017). Thus, the *Ph1* locus promotes early homologous pairing and avoids homoeologous pairing in wheat.

Only a single *Ph1* deletion mutant (*ph1b)* in CS cultivar as described above has been used over the last 40 years to introgress wild relative chromosome segments into wheat. More recently, new chemically induced mutant lines selected for a mutation in *TaZIP4-B2* within the *Ph1* locus have been released (Rey *et al.,* 2017). These lines showed high levels of homoeologous crossovers when crossed with wild relatives. The exploitation of such *Tazip4-B2* mutants, rather than mutants with whole *Ph1* locus deletions, may therefore improve introgressions of wild relative chromosome segments into wheat (Rey *et al.,* 2017).

1.7.2 Other *pairing homoeologous* genes in wheat

Besides the main suppressor of homoeologous pairing gene *Ph1* in wheat, other minor genes were identified. A gene with an intermediate effect was located on the short arm of chromosome 3D and designated as *Ph2* (Mello-Sampayo, 1971). A third suppressor, even less effective than *Ph2*, was located on the short arm of chromosome 3A (Driscoll, 1972; Mello-Sampayo and Canas, 1973). Because of the redundant functional activity of these two minor suppressors located on 3AS and 3DS and their location on homoeologous arms, it was proposed that they are homoeologous loci. Another two additional minor suppressors with a similar effect to that of 3AS were reported on chromosome

4D (Driscoll, 1973) and chromosome arm 2DL (Ceoloni *et al.*, 1986). The level of homoeologous pairing in the double mutant *Ph1*/*Ph2* is very similar to that of the single mutant *Ph1* (Ceoloni and Donini, 1993). This suggests no additive cooperation between both pairing suppressor genes. Because *Ph1* is hemizygous-effective, a single dose is fully functional in suppressing homoeologous pairing in polyhaploids of both bread wheat (Jauhar *et al.*, 1991) and durum wheat (Jauhar *et al.*, 1999).

1.8 Wheat genotyping

1.8.1 The evolution of wheat molecular markers

The early generation of molecular markers developed in the mid-1980s was restriction fragment length polymorphism (RFLP), which required a large amount of high purity DNA to run. RFLPs are no longer used nowadays because more efficient genotyping methods emerged and many RFLPs have been replaced by or converted to polymerase chain reaction (PCR)-based methods. Several prevailing PCR-based marker systems were developed a few years later such as random amplified polymorphic DNA (RAPD) markers. Unfortunately, these markers were later found to be unreliable and unrepeatable. Amplified fragment length polymorphism (AFLP) markers appeared in the mid-1990s with improved reliability and reasonable efficiency (Yang *et al.*, 2015a). In bread wheat, the RFLP, AFLP, and RAPD marker systems detected only low levels of intraspecific polymorphism (Penner *et al.*, 1995; Paull *et al.*, 1998; Kim and Ward, 2000; Hazen *et al.*, 2002) especially in comparison with the simple sequence repeat (SSR) markers that were consistently found to be more polymorphic, easily visualized, stable, and co-dominant (Akkaya *et al.*, 1992; Roder *et al.*, 1995; Powell *et al.*, 1996; McCouch *et al.*, 1997; Song *et al.*, 1999, 2004). However, the development of SSR markers in wheat was difficult and time-consuming due to its large genome size, polyploidy, and the high level of repetitive genome sequences.

In the early 2000s, SSR markers gave way to diversity array technology (DArT) markers increasing the volume of markers for genotyping (Wenzl *et al.*, 2006, Yang *et al.*, 2015). DArT simultaneously genotypes several thousands of loci in a single assay, generating a whole-genome fingerprint by scoring the presence versus absence of DNA fragments in samples of genomic DNA

(Jaccoud *et al.,* 2001). It was successfully used in genetic mapping and fingerprinting studies in wheat (Akbari *et al.,* 2006; Semagn *et al.,* 2006). The genome size of hexaploid wheat, which is about 17 Gb with a high percentage of repetitive DNA (>80%), has resulted in a lack of robust, tightly linked markers for many genes or QTLs for use in breeding programs (Wanjugi *et al.,* 2009; Wang *et al.,* 2015).

The advances in next-generation sequencing (NGS) technology provided the required throughput to conquer the enormous size of the wheat genome. The NGS technology has significantly reduced the amount of time for marker discovery (Xu and Crouch, 2008). Hence, the discovery of single nucleotide polymorphism markers (SNPs) by whole genome sequencing (Berkman *et al.,* 2012; Chia *et al.,* 2012; Xu *et al.,* 2011). SNP refers to a single base change in a DNA sequence, with a usual alternative of two possible nucleotides at a given position (Vignal *et al.,* 2002). SNPs are now the preferred type of markers as they are highly abundant across the entire genome, relatively low cost, locus-specific and highly polymorphic compared to previous molecular marker systems (Varshney *et al.,* 2014; Yang *et al.,* 2015a). Several high-throughput wheat SNP arrays such as 9K (Cavanagh *et al.,* 2013) and 90K (Wang *et al.,* 2014) arrays from Illumina have been developed and facilitated wheat genomics research (Edae *et al.,* 2017). These wheat SNP arrays represent a powerful tool for studying genomic patterns of diversity, inferring ancestral relationships between individuals in populations and studying marker–trait associations in mapping experiments (Wang *et al.,* 2014).

In 2011, the emergence of the genotyping-by-sequencing technique to score SNPs provided a large number of markers (Elshire *et al.,* 2011). The GBS technique works by digesting genomic DNA with restriction enzymes and sequencing short fragments (usually ~100bp) up and downstream of the restriction site. The ligation of unique pairs of DNA barcodes allows large scale multiplexing of individuals when sequencing which are then separated post sequencing bioinformatically. The ability to multiplex multiple samples reduces the cost significantly compared to whole genome sequencing. The availability of reference genomes, including the recently released *T. aestivum* cv. Chinese Spring and *T. turgidum* ssp. dicoccoides cv. Zavitan genome sequences (Avni *et al.,* 2017) allows the sequence reads to be aligned and SNPs called in the

individuals with respect to the reference sequence. Although the results generated by the wheat SNP arrays are generally of a higher quality compared to those generated by GBS (results typically contain a large percentage of missing data points), the cost per sample is substantially higher than GBS (Elbasyonia *et al.,* 2018). The other advantage of GBS is that it can be used for plant species lacking a complete linkage or physical map (Poland *et al.,* 2012).

More recently, the SNPs from arrays have been converted into Kompetitive allele-specific PCR (KASP) markers (Semagn *et al.,* 2014; Thomson, 2014) or semi-thermal asymmetric reverse PCR (STARP) markers (Long *et al.,* 2017). The KASP method, is an improved allele-specific PCR developed by LGC (Middlesex, UK; http://www.lgcgroup.com), shows improved accuracy in allele discrimination and is commercially applied to SNP genotyping. STARP is a novel SNP genotyping method where the genotyping assay is performed under unique PCR conditions using two universal priming element-adjustable primers (PEA-primers) and one group of three locus-specific primers: two asymmetrically modified allele-specific primers (AMAS-primers) and their common reverse primer (Long *et al.,* 2017). It is expected that the STARP technique, with the major advantages of simple assay design, flexible throughputs, high accuracy, and low operational costs, will be applied increasingly in marker assisted selection (MAS) and genetic mapping (Long *et al.,* 2017).

1.8.2 Importance of molecular markers in wheat breeding

Plant breeding essentially includes two major activities: the creation of genetic variation and the selection of beneficial traits. Marker development, in association with agronomic traits, is crucial for the transition from crop genomics to breeding practice (Yang *et al.,* 2015a). Molecular tools are extensively used not only for the construction of molecular maps, but also for identification/isolation of genes for traits of interest. Markers are very useful for MAS in wheat breeding programs to help breeders accelerate the selection process towards the development of improved wheat varieties. Studies have shown that genomics and MAS when incorporated into breeding programs often result in a near two-fold rate of genetic gain compared to standard phenotypic selection (Eathington *et al.,* 2007; Battenfield *et al.,* 2016). Marker-based

molecular breeding methods include marker-assisted germplasm evaluation, marker-assisted backcrossing, marker-assisted recurrent selection, and marker-assisted gene pyramiding (Xu, 2010; Varshney *et al.*, 2012).

With the continuous progress in the development of markers technologies for wheat breeding, several high throughput platforms are now well established. Different genotyping require different platforms. For genetic mapping, the estimation of genetic diversity and whole genome selection, the multiplex high-density SNP arrays and GBS sequencing methods can be used (Rasheed *el al.*, 2016). Both technologies provides important information for fine mapping, gene cloning, and genomic selection (Wang *et al.*, 2014; Zhao *et al.*, 2014). For the genotyping of a small number of specific loci for gene identification, line evaluation, or MAS, a more flexible genotyping platform is suitable to achieve high throughput results at low cost, such as the KASP platform (Semagn *et al.*, 2014; Thomson, 2014). Genotyping-by-sequencing can generate numerous SNP markers covering a high percentage of the genome in a cost-effective manner (Elshire et al., 2011; Poland and Rife, 2012). Therefore, these genome-wide SNPs can be used in genomic selection, genome-wide association study (GWAS) and genetic diversity studies. GWAS provide the means to speed up the production of resistant varieties through marker-assisted selection (MAS) or other biotechnological approaches. Single nucleotide polymorphism (SNP) markers identified in GWA studies can be used to produce efficient tools for MAS in breeding, such as KASP markers.

1.9 Cytogenetics techniques and their application in wheat breeding

Cytogenetic techniques have contributed greatly to the understanding of genetics, biology, reproduction, and evolution studies. From early studies in basic chromosome behaviour, molecular cytogenetic techniques, such as *in situ* hybridization (ISH) introduced by Gall and Pardue in 1969, opened a new era of research combining cytogenetics and molecular biology. Cytogenetics can be applied at different phases of plant breeding and in different ways for rapid and efficient plant improvement. Single colour (sc) or multicolour (mc) fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) represent two special types of cytogenetic techniques that have been of a great use in wheat breeding. Technically, both GISH and FISH experiments

have several steps in common, including chromosome preparation, probe labelling, blocking DNA preparation, target-probe DNA hybridization, post-hybridization washes, and hybridization signal detection (Xu *et al.*, 2016).

1.9.1 Fluorescence *in situ* hybridization technique: FISH

FISH is a type of hybridization that uses a labelled DNA or RNA strand as a probe to localize a specific DNA or RNA sequence in a portion or section of tissue (*in situ*) or in the entire tissue (Devi *et al.*, 2005). This technique is generally used to locate the physical position of a known DNA sequence on a chromosome. FISH is most frequently performed on mitotic metaphase chromosomes due to their ease of availability (Trask and Trask, 1999). Typically, metaphase chromosomes are accumulated through pre-treatment with mitotic spindle inhibitors such as colchicine. FISH uses synthetic polynucleotide strands that bear sequences known to be complementary to specific target sequences at specific chromosomal locations. The polynucleotides are bound via a series of linked molecules to a fluorescent dye (Xu, 2010). The sites located will exhibit fluorescence and can be photographed with a fluorescent microscope. Thus, precise physical location of genes or DNA can be visualized on chromosomes. A variety of probe-labelling schemes are now available for simultaneous detection of two or more sequences in the same nucleus. Multicolour FISH (mc-FISH) takes advantage of simultaneous hybridization of several DNA probes labelled by different fluorochromes to different targets on the same chromosome sample. Hybridization signals from different probes are detected using different fluorescence filter sets. This technique is extremely useful for identifying chromosomes within a species or detecting intergenomic chromosome rearrangements in a polyploid species (Dechyeva and Schmidt, 2016; Komuro *et al.*, 2013; Liu *et al.*, 2018).

FISH has been utilized in many plants to identify chromosomes accurately, using species-specific repetitive sequences, ribosomal genes and even unique sequences. In wheat, some tandemly repeated sequences, such as pTa-535, pTa-713, and pTa-86, are used as FISH probes to identify the A, B-, and D-genome chromosomes (Komuro *et al.*, 2013). In fact, these mc-FISH probes allow the development of a wheat karyotype, making possible the identification of every chromosome group of every genome. FISH represents a powerful tool for

investigating genome homology between polyploid species and their diploid progenitors. Using specific mc-FISH probes on good quality mitotic spreads of *Thinopyrum bessarabicum* disomic addition lines in wheat, Grewal *et al.* (2018) were able to develop a FISH karyotype for *Th. bessarabicum.* Allocation of each of the seven pairs of the alien chromosomes to *Triticeae* homoeologous groups was possible based on visual characteristics of mc-FISH patterns. Multicolour-FISH has also been used to detect rye (*Secale cereale* L.) chromosomal fragments in wheat-rye hybrids (Tsuchida *et al.,* 2008; Zhuang *et al.,* 2010; Fu *et al.,* 2013). *S. cereale* clone pSc119.2 mainly hybridizes to B-genome chromosomes of wheat and the R-genome of rye, and *Ae. tauschii* clone pAs1 produces signals especially on D-genome chromosomes. Using these probes together allows the identification of most wheat chromosomes and all rye chromosomes (Schneider *et al.,* 2003; Contento *et al.,* 2005; Tang *et al.,* 2014).

1.9.2 Genomic *in situ* hybridization technique: GISH

GISH, which involves the use of the total genomic DNA from one species as a probe (Stace and Bailey, 1999), enables the observation of the respective genomes of each species present in a hybrid, as well as the observation of whether chromosomal recombination is occurring in different generations of hybrid progeny (Silva *et al.,* 2013; Türkösi *et al.,* 2016). GISH can be used as single colour (sc-GISH) highlighting only one genome or in multicolour (mc-GISH) to differentiate chromosomes from different genomes in polyploid species (Zhang and Friebe, 2009). In hexaploid wheat, the total genomic DNA of *T. urartu* (A-genome progenitor) and *Ae. tauschii* (D-genome progenitor) labelled with two different colour probes using nick translation procedure that enables a high incorporation of dNTP-fluorophores, and non-labelled total genomic DNA of *Ae. speltoides* (possible B-genome progenitor) were hybridized *in situ* to metaphase chromosome spreads (King *et al.,* 2017). This mc-GISH has been used to distinguish the three A- B- and D-genomes of wheat. In case of intergenomic rearrangement between the different genomes, the translocation breakpoints can be easily determined (Schwarzacher *et al.,* 1992).

GISH offers new opportunities in phylogenetic and taxonomic studies for determining and testing the genomic relationships of wild and cultivated plant species and gives unique information about similarities between DNA from

related species. GISH is extensively used for determining chromosome constitutions of amphiploids, identifying alien chromosomes in amphidiploids, chromosome addition or substitution lines, and determining the size and location of the alien chromosome segments in introgressions lines (Cai *et al*., 1998; McArthur *et al.*, 2012). Both sc-GISH and mc-GISH allowed the identification of several alien introgressions into hexaploid wheat and helped trace the introgressed segments through the subsequent generations of back crossing for *Amblyopyrum muticum* (King *et al.*, 2017), *Aegilops speltoides* (King *et al.*, 2018) and *Thinopyrum bessarabicum* (Grewal *et al.*, 2018). Furthermore, mc-GISH was used to discriminate the E genome of *Thinopyrum ponticum* in wheat/ *Th. ponticum* addition, substitution and translocation lines (Fu *et al.*, 2012), and to characterize the constitution of five partial amphiploid lines derived from wheat/*Thinopyrum intermedium* hybridizations (Han *et al.*, 2004).

1.10 The importance of phenotyping in wheat breeding

Effective use of genetic resources in breeding programs requires evaluation of the diversity in the genepool and characterization of available accessions in order to detect the presence of variants of potential traits of interest for breeding (Nazco *et al.*, 2012). Thus, efficient phenotyping techniques are essential to develop new wheat varieties with higher yield potential. A plant phenotype is a set of structural, morphological, physiological, and performance-related traits of a given genotype in a defined environment (Granier and Vile, 2014). The phenotype results from the interactions between a plant's genes and environmental (abiotic and biotic) factors. Plant phenotyping involves a wide range of plant measurements such as growth development, canopy architecture, physiology, disease and pest response, and yield. The recent advances in sequencing technology have facilitated the ability to generate high quality genotypic data for a large number of individuals. In this case, the availability of accurate phenotypic data is extremely important and plays a key determinant for the success of association studies between genotypes and phenotypes such as QTL mapping or genome wide association studies (GWAS). Most importantly, all of the marker data, obtained or used, must be associated with carefully measured phenotypes to establish the real value of the marker(s). In

addition, the environment in which plants are grown, may change quite often and the first indication of these changes is the phenotype. Hence plant breeders must always pay attention to the phenotype.

1.10.1 Conventional wheat phenotyping

Conventional wheat phenotyping is based on visual selection. For some traits, visual phenotyping remains a remarkably effective way for handling massive populations of experimental lines that are generated in a breeding program. Wheat phenotyping is carried out either under natural field conditions or under controlled conditions such as glasshouses and growth chambers. Experiments carried out under controlled conditions help the breeding process, for example, where screening can be used for limited amounts of material or if the screening requires a specific race or isolate of a pathogen. This process can speed up breeding and gives an idea of the disease reaction at the seedling stage as well as at the adult stage.

For biotic stress, breeders need to decide the degree of resistance that is acceptable and select only plants with at least that level of resistance. Different recording scales are used in wheat phenotyping for biotic stresses, depending on the pathogen life cycle, the affected part and the growth stage of the plant. For example, leaf and stem rust diseases caused by *Puccinia triticina* and *Puccinia graminis tritici* respectively, are among the most important wheat diseases. Under field conditions, the leaves or stems are compared with diagrams on which various percentages of the area have been covered with spots of various sizes and represent pustules. Based on the size of pustules and the associated necrosis or chlorosis, infection responses are classified into four discrete categories: R= resistant, MR= moderately resistant, MS= moderately susceptible, and S= susceptible (Roelfs *et al.*, 1992). A different scoring scale is used at seedling stage. Seedling scoring in this case follow the 0-4 scale as described by Roelfs *et al*. (1992). Another example is Fusarium head blight (FHB) caused by *Fusarium graminearum.* This pathogen occurs worldwide making it one of the most dominant, widespread and destructive pathogens. It directly affects the grain causing it to shrink and wrinkle inside the head and the accumulation of harmful mycotoxins. Symptoms are visible on the spikelet within the wheat spikes. Two major types of resistance to FHB were identified

(Schroeder and Christensen, 1963). Under greenhouse or field conditions, the evaluation of FHB severity is rated visually as the number of infected spikes per plot (type I resistance) and the number of infected spikelets per spike (type II resistance) (Voss *et al.*, 2010; Miedaner *et al.*, 2014).

For abiotic stresses, heat and drought are two of the major stresses constraining wheat productivity worldwide and causing significant yield losses (Fischer and Maurer, 1978; Prasad *et al.*, 2011). Both stresses are more likely to occur simultaneously in semi-arid and hot growing regions (Tricker *et al.*, 2016). Yield penalty is associated with long periods of drought coinciding with heat waves above 32°C during heading and grain filling stages (Wardlaw and Wrigley, 1994). Phenotyping remains a key for screening and selecting improved breeding materials. In the selection for drought and heat tolerance, flowering time and earliness of wheat plants are considered important. Flowering time is recognized to be the most critical factor-affecting yield under drought conditions. For instance, reduced number of days to heading and days to maturity may contribute to drought escape. Consequently, earliness is also important for breeding for drought stress tolerance (Lopes *et al.*, 2012). In glasshouse trials, it is possible to study the parameters considered important in the field. Relative yield performance of genotypes under water stressed and non-stressed environments also can be used as an indicator of drought tolerance (Mohammadi, 2016). Thus, phenotyping using controlled water regimes offers yield based germplasm screening, allowing for selection of high yielding genotypes under both stressed and non-stressed conditions (Mwadzingeni *et al.*, 2016).

1.10.2 High throughput phenotyping

An ideal phenotyping method must be accurate, high throughput, reproducible and be easy to use. Recently several field-based high-throughput phenotyping platforms have been developed (Crain *et al.*, 2018) due to the rapid development and decreasing cost of computers and digital cameras this is no longer an issue. High throughput phenotyping platforms could provide the keys to connecting the genotype to phenotype by both increasing the capacity and precision of phenotyping and also reducing the time to evaluate huge plant populations. Much of what is currently considered high-throughput

phenotyping is based on remote sensing. The most common types of remote-sensing devices used for crop phenotyping include multispectral, hyperspectral, fluorescence, and thermal sensors (particularly for ground-based phenotyping platforms), or imagers (which may be deployed from aerial platforms using the radiation reflected or emitted by the canopy; Araus *et al.,* 2018). Remote-sensing tools allow assessment of physiological yield components that are clearly and conceptually related to crop productivity and stress adaption in terms of resource acquisition (radiation, water, nutrients, etc.), resource use efficiency, or downstream biomass partitioning (Araus and Cairns, 2014; Deery *et al.,* 2014; De Souza *et al.,* 2017).

Digital image analysis based phenotyping of plant diseases can be more accurate than human estimates in a number of pathosystems (Bock *et al.,* 2008; Kokko *et al.,* 2000; Martin *et al.,* 1998; Xie *et al.,* 2012). The ability to write batch processing macros in image processing software such as ImageJ allows multiple images to be analysed in a single step. For instance, in order to gain accurate phenotypic data for STB disease on a large scale, a high throughput phenotyping method was developed based on automated digital image analysis that accurately measures the percentage of leaf area covered by lesions as well as pycnidia size and number (Stewart and McDonald, 2014). A seedling inoculation assay was conducted using 361 *Z. tritici* isolates originating from a controlled cross between two different winter wheat cultivars. The results showed that pycnidia size and density to be quantitative traits with a continuous distribution in the progeny (Stewart and McDonald, 2014). Precise measures of percentage leaf area covered by lesions provided a quantitative measure of host damage. Thus, digital image analysis has the ability to produce the accurate phenotype data required by a QTL mapping project.

1.11 Septoria tritici blotch disease of wheat

1.11.1 Disease symptom

Z. tritici (teleomorph *Mycosphaerella graminicola*) (Quaedvlieg *et al*., 2011), previously named *Septoria tritici*, is a hemibiotrophic pathogen characterized by two infection stages. In the early infection process, the pathogen behaves as a biotrophic parasite. Through penetration via host stomatal cavities, the pathogen derives its nutrition from the apoplast around

living cells. Thus, the initial symptoms are small chlorotic spots on the leaves (Steinberg, 2015). During the later stage of infection, leaf necrosis caused by toxic compounds produced by the fungus during the intercellular colonization become apparent (Kema *et al*., 1996a). At this stage, *Z. tritici* behaves as an aggressive necrotrophic in an infection phase that leads to the appearance of characteristic lesions bearing dark brown or black spots that are the fruiting structures on the leaf surface as a sign of the disease (**Figure 1-2**). Lesions on mature leaves are most often long, narrow and delimited by leaf veins but can also show irregular or elliptical shapes. Under high infection, lesions caused by *Z. tritici* tend to coalesce and form large necrotic spaces on the leaf surface. On susceptible wheat genotypes and under high infection levels of *Z. tritici* the whole leaf can become necrotic. Infections on the flag leaves can cause the most severe losses by reducing grain weight.

Figure 1-2. Septoria tritici blotch disease symptom on durum wheat leaf (Tunisia, 2018).

1.11.2 *Z. tritici* reproduction

Z. tritici has asexual and sexual reproduction cycles. The asexual reproduction is produced in pycnidia, specialized structures harbouring asexual conidia/pycnidiospores spores (**Figure 1-3A**). Pycnidiospores are exuded from the pycnidia in cirrhi (**Figure 1-3B**) that are usually a milky white colour (Ponomarenko *et al*., 2011). During the growing season, many cycles of asexual reproduction can occur.

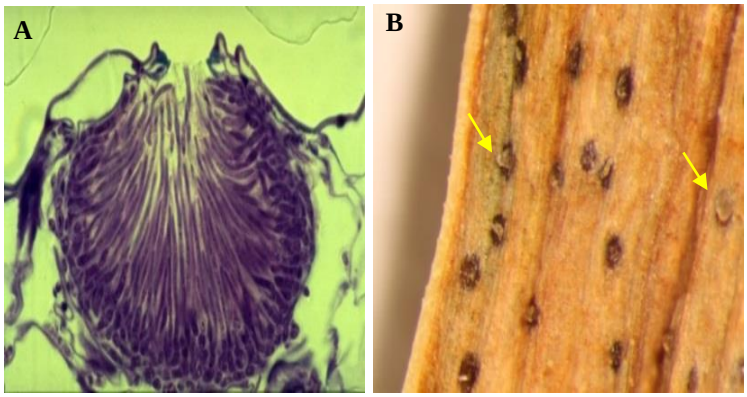

Figure 1-3. Asexual reproduction structures of *Z. tritici*: A. Pycnidia filled with pycnidiospores (Kema G.H.J., copyright); B. Cirrhi exudation through pycnidium (yellow arrows).

The fructification structures in the sexual cycle are called pseudothecium harbouring several asci that contain eight ascospores each (**Figure 1-4A**). The sexual cycle of *Z. tritici* is driven by its heterothallic bipolar mating system. In fact *Z. tritici* is characterized by two *MAT* alleles at a single locus designated as *MAT1-1* and *MAT1-2* (Kema *et al.*, 1996b). The sexual ascospores (**Figure 1-4B**) result from the mating between isolates with opposite mating types (*MAT1-1* and *MAT1-2*) required for sexual reproduction (Kema *et al.*, 1996b).

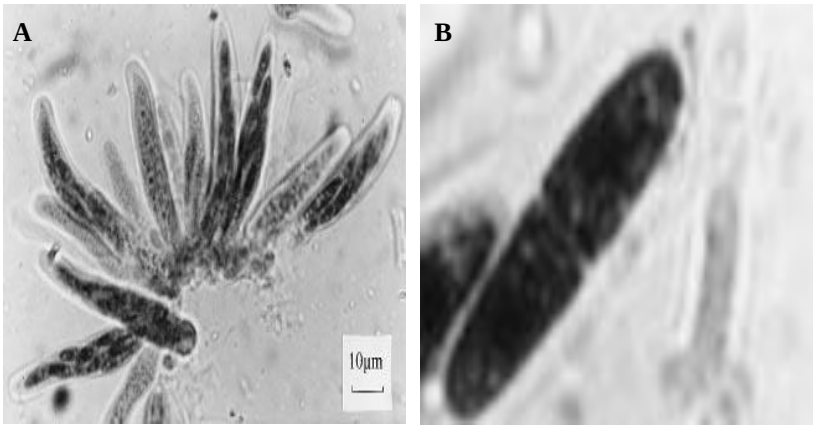

Figure 1-4. Sexual reproduction structures of *Z. tritici*: A. Asci of *Z. tritici* containing 8 ascospores each. B. Ascospore. (Halama, 1996).

1.11.3 STB disease cycle

In the Mediterranean Basin where wheat is sown in November and harvested in July, the cycle of STB disease usually becomes apparent between February and May provided that climatic conditions are favourable for pathogen development particularly with high relative humidity (85%), an optimum temperature of 22°C and free water on the leaves that are important for pathogen growth (Eyal *et al.*, 1987). Disease infection starts at the seedling stage by a

primary inoculum that can be initiated either by air-borne ascospores or splash-dispersed pycnidiospores (Eyal *et al.,* 1987). In fact, *Z. tritici* can survive between seasons as asexual and sexual spores in their respective conservation structures on either wheat debris, wheat volunteers that grows out of the falling seeds during harvest or an alternative host (**Figure 1-5**). Pycnidia were reported to be found in wheat stubble and debris in the soil between wheat seasons (Hilu and Bever, 1957; Suffert *et al.,* 2011). The capacity of *Z. tritici* to infect other grasses has been demonstrated as well, suggesting that grass weeds may act as disease foci, which can possibly explain the early wheat infections. Kema *et al.* (1996c) were able to collect pseudothecia on wheat volunteer that grows in the field from the seed falling into the ground after harvest. Wheat volunteer plays an important role in the survival of *Z. tritici* between seasons and provides a source of primary inoculum for the next wheat season. Furthermore, produced in a mucilaginous matrix (cirrhus), pycnidiospores can remain viable during extended periods of dry weather. They are usually considered as a major source for the primary inoculum of *Z. tritici* during the early infection period (Shaw and Royle, 1989).

Secondary inoculums are produced during the growing season (**Figure 1-5**). Leaves bearing pycnidia are produced roughly 14 to 40 days after infection, depending on the host and seasonal conditions. Pycnidiospores are generally dispersed through rain-splashing (Shaw and Royle, 1993). The secondary spread of STB can be ensured by the sexual stage of *Z. tritici* as well. Both pycnidiospores and ascospores contribute to the epidemic but the asexual cycle seems to dominate during the growing season. Ascospores can be airborne over large distances, while pycnidiospores are unlikely to disperse far from their point of infection. Several cycles of sexual and asexual reproduction can occur during the growing season to result in various levels of infection.

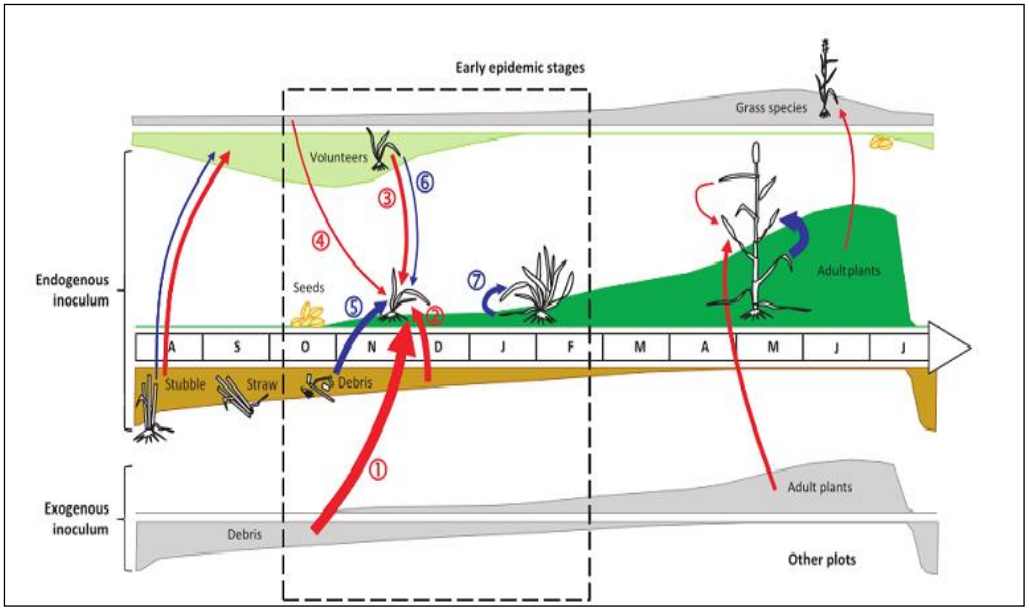

Figure 1-5. A schematic representation of the dynamic of a STB epidemic (Suffert *et al.*, 2010): Red arrows 1, 2, 3, and 4 represent the wind-dispersal of the ascospores as a primary inoculum. Blue arrows 5, 6, and 7 represent the splash-dispersed of the pycnidiospores as a secondary inoculum.

1.11.4 *Z. tritici* pathogenic dimorphism

Z. tritici pathogen express a pathogenic dimorphism to wheat species to either *T. aestivum* or *T. durum;* durum wheat specific isolates can infect only durum wheat and the same for the bread wheat while some isolates have the ability to infect both of the species. This specificity of the pathogen within wheat species has been reported in many countries (Eyal *et al*., 1973; Prestes and Hendrex, 1978; Kema *et al*., 1996a; 1996b; Zhan *et al*., 2004; Ware, 2006). For instance, in Tunisia *Z. tritici* affects only durum wheat while no disease symptoms could be found on any of the bread wheats. In other countries such as Morocco, only bread wheat is affected. However, in certain countries such as Algeria, *Z. tritici* isolates have the ability to affect both species. Genetic studies of the pathogen showed that both bread wheat and durum wheat isolates are sexually compatible and can generate viable off-spring that show recombinant pathogenicity towards both wheat species (Ware, 2006; Wittenberg *et al*., 2009).

1.11.5 Genetic structure of *Z. tritici*

An understanding of the genetic structure of pathogen populations can provide important insights on the evolutionary processes that shaped the existing population structures and may allow prediction of the evolutionary

potential of the pathogen in the future (Drabešová *et al.,* 2012). Genetic studies of *Z. tritici* populations using molecular markers showed high levels of genetic diversity at different spatial levels (McDonald and Martinez, 1990a; 1990b; 1991a; 1991b). Among 711 isolates analysed from one field in Oregon, USA, 654 distinct genotypes were identified (Boeger *et al.,* 1993; Zhan *et al.,* 2003). Therefore, a high level of genetic diversity is not only seen in a worldwide population but also within-field (Berraies *et al*., 2013b) as well as within individual leaf disease lesions (Linde *et al.,* 2002). The high level of genetic diversity of this pathogen is consistent with high levels of sexual recombination and supports the epidemiological studies that implicate ascospores as the primary inoculum (Shaw and Royle, 1989; Zhan *et al.,* 2001) and secondary inoculum during the course of the season (Zhan *et al.,* 2001). Moreover, the equal distribution of both mating type idiomorphs (*MATI-1* and *MATI-2*), found in most populations worldwide, is consistent with the active sexual reproduction of the pathogen during the growing season (Waalwijk *et al.,* 2002; Zhan *et al.,* 2002; Siah *et al.,* 2010). In *Z. tritici* populations, genotypes are short-lived due to the occurrence of the sexual cycles (Wittenberg *et al.,* 2009). Sexual recombination generates genetic variation that allows faster adaptation to fluctuating and/or stressful environments. The analysis of the frequency of recombination of two separate populations from a moderately resistant plant host "Madsen" and a susceptible host "Stephens" by Zhan *et al.* (2007) showed that 13% of the isolates sampled from the resistant host were recombinants, compared with 9% in the samples collected from the susceptible host. The pathogen isolates originating from the resistant cultivar displayed higher levels of fitness, virulence and fungicide tolerance than those originating from the susceptible cultivar which confirmed the hypothesis that sexual reproduction facilitates the evolution of parasites to overcome host resistance (Zhan *et al.*, 2007).

1.11.6 Mechanism of resistance of wheat to pathogens

Three main strategies are diploid by pathogens to infect plants: necrotrophy, biotrophy, or hemibiotrophy (Cooke *et al.,* 2006). Necrotrophy is defined as growth and nutrition of the pathogen on dead or dying plant material. In this mode of infection, death of host plant cells precedes or accompanies

colonization of the pathogen. Cell death is often induced by toxins and/or enzymes targeted to specific substrates. Biotrophic and hemibiotrophic pathogens invade living cells and subvert metabolism to favour their growth and reproduction. Biotrophic pathogens colonize the host plant and suppress defence responses without any visible symptoms seen in the host plant. Hemibiotrophic pathogens instead, first establish a biotrophic interaction with the host plant but at later infection stages, switch to necrotrophy. Generally, biotrophs tend to cause disease on only one or a few related plant species. In contrast, hemibiotrophic fungi such as *Z. tritici* kill surrounding host cells during the later stages of the infection (Kema et al., 1996a).

Effectors are pathogen-produced molecules that have a specific effect on one or more genotypes of a host plant. Effector are able induce or suppress a response in the plant. Effectors of biotrophic and hemibiotrophic pathogens typically include Avirulence (Avr) proteins that are recognized by matching resistance (R) proteins. In the absence of the R protein, the effectors promote virulence by suppressing innate immune responses, such as pathogen-associated molecular patterns (PAMP)-triggered immunity, in a process termed effector- triggered susceptibility (ETS), which allows the establishment of a biotrophic interaction (Jones and Dangl, 2006). Hence, plant resistance can be achieved via the loss or alteration of the toxin's target or through detoxification. In the case of hemibiotrophic pathogens, effectors to suppress cell death are produced in the intial phases of infection. At later phases, these are downregulated and other necrotrophic effectors are induced and dominate the interaction.

Host resistance to these pathogens is typically governed by R genes encode R proteins protein that following pathogen recognition, the R protein is presumed to activate signalling cascade(s) that coordinate the initial plant defense responses to impair pathogen ingress.

In the case of *Z. tritici* it was seen that R gene coding for PRR- like receptor-like proteins recognize fungal secreted effectors in the plant apoplastic space.

Plant cell wall degrading enzymes (PCWDEs) produced by the pathogen or some of their degradation products may also act as effector molecules that elicit defense responses in plants (Esquerre´-Tugaye´ *et al.* 2000). PCWDEs can be recognized by the plant immune

system and become targets of inhibitory proteins (Federici *et al.* 2006; Juge 2006) produced by the host plant during the biotrophic and/or necrotrophic stages.For instance, in the case of Z. tritici the enzymes cutinase, a cellulase, and a hemicellulase are produced at very low expression during the biotrophic phase to avoid recognition by the plant immune. However, their transcription increase approximately 10 to 100-fold during the necrotrophic stage.

1.11.7 Methods of STB disease control

1.11.7.1 The cultural practice

The cultural practice strategy effectively contributes to the control of STB using methods such as tillage, crop rotation, late sowing dates and the use of balanced fertilizers. Crop rotation can reduce the quantity of the primary inoculum present in the stubble. Moreover, the use of lower levels of N fertilization associated with lower sowing densities could potentially decrease the susceptibility of wheat (Saulas and Meynard, 1998). This strategy, while it can reduce the epidemic level, is not effective to control the disease.

1.11.7.2 The biological control

The biological control in general is the use of microorganisms known to be competitive with, or antagonistic to pathogens of the crop being grown. Nolan and Cooke (2000) presume that the slow infection process and long latent period associated with *Z. tritici* pathogenesis of wheat may provide a window of time during which microorganisms, and/or their biochemical products might disrupt STB development. In this context, it has been shown that some bacteria have the ability to produce an antifungal substance. For instance, Flaishman *et al.* (1996) presented evidence that the hydrogen cyanide (HCN) produced by the bacterium *Pseudomonas putida* (strain BK8661) can suppress STB development. In another study at small scale field trials using the bacteria *Bacillus megaterium* (strain MKB135), STB development has been reduced by up to 80% (Kildea *et al*., 2008). Some fungi can also be used to control STB disease. Perellò *et al*. (2006) showed that a number of *Trichoderma spp*. isolated from different sources, including wheat phylloplanes, were able to control STB throughout the growing season under field conditions. Despite the effectiveness of this method, no biological agents have yet been registered for the control of this disease.

1.11.7.3 The chemical control

Fungicide treatment is currently the most effective method to control STB particularly when cultivated wheats lack effective resistance. Two major categories of fungicides have been commonly used to control STB: the quinone outside inhibitors (QoI) also known as strobilurins and the sterol demethylation inhibitors also known as azoles. In addition, benzimidazole fungicides have also been used. The use of fungicides is becoming less effective due to the development of fungal populations resistant to some of the fungicides (Drabešová *et al*., 2012). Strobilurin and triazole are costly and have become less effective due to development of a high degree of resistance of *Z. tritici* populations spread throughout many European countries (Fraaije *et al*., 2005; 2007; Cools and Fraaije, 2008). The application of multi-site fungicides, such as chlorothalonil, and SDHI fungicides is progressively replacing the use of stobilurin and triazoles (Torriani *et al*., 2015).

1.11.7.4 The host resistance

Host plant resistance is considered as the best strategy of STB management. The use of resistant cultivars reduces the necessity to apply fungicide treatments and provides a reliable strategy for disease control. This strategy is considered as the most economical and environmentally safe method for disease control and subsequently enhancing crop production. Improving cultivar resistance to STB can be achieved by breeders through the integration of resistance genes into, or the elimination of dominant susceptibility genes from wheat cultivars. Reduction of areas grown with susceptible varieties could ensure adequate levels of disease control during the season and also provide farmers with better newly developed high yielding resistance varieties to ensure a better and sustainable production.

1.11.8 *Z. tritici*-wheat interaction

In contrast to the genetic variability of the pathogen, much less is known about variability for pathogenicity within the pathogen populations. The susceptibility of a particular wheat cultivar to a given *Z. tritici* isolate can be determined by consideration of the degree of lesion coverage on the leaf (Rosielle, 1972), pycnidial density (Eyal and Brown, 1976) and disease height (Eyal *et al*., 1987). Although isolates do differ in virulence, to date there is no

evidence of clear distinct physiologic races. To further understand the compatibility and incompatibility reactions of *Z. tritici* on wheat, proteomic analysis of leaf apoplastic fluid during the biotrophic stage of infection during the two types of reactions have been investigated (Yang *et al*., 2015b). At this stage of infection, *Z. tritici* needs to acquire apoplastic nutrients, shape the plant cell structures, and overcome the activated apoplastic defences to survive via the secretion of effectors involved in the detoxification of defence-related molecules as well as protection against recognition by the plant (Kema *et al*., 1996b). At this point the interaction between the pathogen effectors and the defence mechanism of the wheat plant can lead either to a compatible or an incompatible interaction. It has been shown that a compatible reaction is associated with inactivation of the plant apoplastic responses by the fungus and its defences to oxidative stress, as well as the perturbation of the plant cell wall during the initial biotrophic stage, followed by the strong induction of plant defences during the necrotrophic stage (Yang *et al*., 2015b). Thus, incompatibility is probably associated with a proteome-level activation of host apoplastic defences as well as fungal inability to adapt to stress and interfere with the host cell at the biotrophic stage of the interaction (Yang *et al*., 2015b).

Analyses of the genetics of the host and the pathogen revealed clear interactions between a resistance gene in the host and the corresponding virulence gene in the pathogen, confirming the existence of gene-for-gene (GFG) interactions in the *Z. tritici*-wheat pathosystem (Brading *et al*., 2002). This GFG interaction implies that *Z. tritici* encodes proteins or metabolites that mediate genotype-specific interactions. Many studies have focused on determination of the virulence genes in *Z. tritici*. In this context, Kema *et al*. (2008) identified several fungal genes that were expressed during the pathogenesis phase. These genes included a number of cell wall degrading enzymes, a broad set of genes involved in signal transduction and a range of ATP-binding cassette and major facilitator superfamily transporter genes potentially involved in protection against antifungal compounds or the secretion of pathogenicity factors. In addition, QTL mapping approaches using next generation sequencing (NGS) technology have been successfully applied in *Z. tritici* to identify 16 novel candidate genes associated with isolate specific virulence (Lendenmann *et al.*, 2014). Furthermore, the completion of the *Z.*

tritici genome sequence by Goodwin *et al*., (2011) facilitated the identification of candidate effector genes, primarily coding small secreted proteins proven to generate symptoms using the infiltration system (Ben M'Barek *et al*., 2015). Some effectors caused intense local lesions whereas others induced spreading lesions, suggesting that these candidate effectors can have avirulence as well as virulence functions.

1.11.9 Genetics of wheat resistance to STB disease

1.11.9.1 *Stb* resistant genes

The genetic resistance to STB in wheat can be either qualitative (Wilson, 1979; Somasco *et al.*, 1996; McCartney *et al.*, 2002) or quantitative. (Jlibene *et al.*, 1994; Simon and Cordo, 1998). Qualitative resistance is controlled by major genes that can be very effective but often short lived and lead to resistance break down due to changes and virulence shifts in pathogen populations. Quantitative resistance is controlled by a combination of major and/or minor genes offering partial resistance. It's more durable and less affected by changes in pathogen populations.

During the last two decades, 20 major genes conferring resistance to *Z. tritici* have been identified (reviewed in Brown *et al.*, 2015) (**Table 1-1**), of which 18 major resistance genes are referred to as *Stb*: *Stb1* to *Stb18* (Chartrain *et al.*, 2009; Goodwin, 2012; Ghaffary *et al.*, 2011; 2012). The resistance genes, chromosome location, source of resistance and the stage of expression are shown in the **Table 1-1**. The first described gene was named *Stb1* by Wilson (1985) and identified in the wheat cultivar Bulgaria 88 by Rillo and Caldwell (1966). *Stb2* and *Stb3* genes were identified in cv. "Veranopolis" and "Israel 493" respectively, in Australia by Wilson (1985). These three genes (*Stb1*, *Stb2* and *Stb3*) were mapped to chromosome arms 5BL (Adhikari *et al*., 2004b), 1BS (Liu *et al.*, 2013) and 7AS (Goodwin and Thompson, 2011) respectively. To date, all *Stb* genes for *Z. tritici* have been identified in bread wheat. While most of them are located in known bread wheat cultivars, some of the genes have been identified in synthetic hexaploid wheat, such as *Stb5*, *Stb8*, and *Stb17* on the cultivars "Synthetic 6X", "Synthetic W7984" and "SH M3" respectively.

Table 1-1. Major STB resistance genes (reviewed in brown *et al.*, 2015).

Locus	Chromosome	Cv. source	Stage*	References
Stb1	5BL	Bulgaria 88	S, A	Adhikari *et al*. (2004a)
Stb2	1BS	Veranopolis	A	Liu *et al.* (2013)
Stb3	7AS	Israel 493	A	Goodwin *et al*. (2015)
Stb4	7DS	Tadinia	S, A	Adhikari *et al.* (2004c)
Stb5	7DS	Synthetic 6X	S, A	Arraiano *et al*. (2001b)
Stb6	3AS	Flame	S, A	Brading *et al.* (2002)
Stb7	4AL	ST6	S	McCartney *et al*. (2003)
Stb8	7BL	W7984	A	Adhikari *et al*. (2003)
Stb9	2B	Courtot, Tonic	S	Chartrain *et* al. (2009)
Stb10	1Dc	Kavkaz-K4500	A	Chartrain *et al.* (2005a)
Stb11	1BS	TE9111	S	Chartrain *et al*. (2005b)
Stb12	4AL	Kavkaz-K4500	S	Chartrain *et al*. (2005a)
Stb13	7B	Salamouni	S	Cowling (2006)
Stb14	3B	Salamouni	S	Cowling (2006)
Stb15	6AS	Arina, Riband	S	Arraiano *et al*., (2007)
Stb16q	3D	SH M3	S, A	Ghaffary *et al.* (2012)
Stb17	5AL	SH M3	A	Ghaffary *et al.* (2012)
Stb18	3AS	Balance	S, A	Ghaffary *et al.* (2011)
StbWW	1BS	WW1842	S	Raman *et al.,* (2009)
TmStb1	$7A^{m}S$	*T. monococcum*	S	Jing *et al.,* (2008)

*S: seedling, A: adult

Out of all the *Stb* genes, the *Stb6* that conferred resistance to *Z. tritici* isolate IP0323, is the only quantitative gene which showed a GFG reaction with IP0323 *Z. tritici* isolate (Brading *et al*., 2002). *Stb6* has been identified in "Flam" and "Hereward" UK cultivars and mapped at the distal end of the short arm of chromosome 3A. The recent map-based cloning of *Stb6* demonstrated that it encodes a conserved wall-associated receptor kinase (WAK)-like protein, which detects the presence of a matching apoplastic effector (Brading *et al.,* 2002; Kema *et al.,* 2018) and confers pathogen resistance without a hypersensitive response (Saintenac *et al.,* 2018).

Another *Stb* gene designated as *StbWW*, identified in three populations in Australia, was mapped on chromosome arm 1BS (Raman *et al.,* 2009) at or near *Stb1, a* gene already identified and mapped in TE9111 (Chartrain *et al.,* 2005a). *Stb2* was also located close to or at the *Stb11* locus (Liu *et al.,* 2013) and thus these genes may all be *Stb11*, which may have spread in global wheat breeding by the movement of elite breeding lines from CIMMYT (reviewed in Brown *et*

al., 2015). Besides the *Stb* genes identified in bread wheat, another major resistance gene in *Triticum monococcum* has also been identified and named *TmStb1,* from accession MDR043 of diploid emmer wheat. This gene has been mapped to chromosome 7AmS (Jing *et al*., 2008).

The reaction of resistance genes to *Z. tritici* depends not only on the virulence and the genetic variability of the pathogen isolates, but also on the physiological stage of the wheat plant. The resistant loci can be effective either at the seedling stage or at the adult stage or at both stages (**Table 1-1**) (Arraiano *et al.,* 2001b; Kema and van Silfhout, 1997). In certain cultivar-by-isolate interactions, adult-plant responses to *Z. tritici* do not necessarily reflect responses of seedlings to the pathogen (Kema and van Silfhout, 1997; Chartrain *et al.,* 2004). *Stb17* is an example of a quantitative adult plant resistant gene which is ineffective at the seedling growth stage (Ghaffary *et al.,* 2012). However, some studies showed that certain qualitative and genotype-specific resistances can be independent of a plant's growth stage (Kema and van Silfhout, 1997; Arraiano *et al.,* 2001a; Brown *et al*., 2001) whereas the expression of the partial resistance depends on the plant's growth stage (Chartrain *et al*., 2004).

1.11.9.2 QTLs for STB disease resistance

Several QTLs conferring resistance to STB have been identified (Kelm *et al.,* 2012; Risser *et al.,* 2011; Kosellek *et al.,* 2013; Adhikari *et al*., 2015). To date, 167 QTLs of resistance against STB have been detected in a total of nineteen bi-parental mapping populations (Brown *et al.,* 2015). All chromosomes, except 5D, carry at least one QTL or meta-QTL for STB resistance. Three chromosome arms, 3BL, 6BS and 7DL, were especially involved in quantitative resistance to STB according to the number of QTLs identified (Brown *et al.,* 2015).

Eriksen *et al*. (2003) reported QTLs on chromosomes 2BL, 3AS, 3BL, 6B, and 7B in a doubled haploid population of a cross between the susceptible winter wheat cultivar "Savannah" and the resistant cultivar "Senat". Miedaner *et al.* (2012) identified five QTL in each of two populations (Arina/Forno, History/Rubens) which explained 45–63% of the genotypic variation. Raman *et al.* (2009) evaluated three double haploid populations derived from Chara/WW2449, Whistler/WW1842, and Krichauff/WW2451 and found that resistance to the pathogen was provided in the three populations by a single

major gene designated as *StbWW2449*, *StbWW1842*, and *StbWW2451* respectively and located on the short arm of chromosome 1B. A new QTL named *QStb.2A* has been identified in the synthetic hexaploid wheat "Lago". This QTL is the first to be mapped on chromosome 2A (Adhikari *et al.*, 2015). In a study of two mapping populations, conducted under field conditions using two resistant bread wheat sources from CIMMYT and evaluated over multiple environments, O'Driscoll *et al*. (2014), confirmed the presence of quantitative resistance to STB in both populations, as well as a number of consistent QTLs across the five environments, located on chromosomes 1BS, 3AL, 5AL and 7AS in both populations (O'Driscoll *et al*., 2014).

1.11.9.3 Resistance genes in durum wheat

Although STB is a severe disease of modern cultivars of durum wheat, especially in North Africa, all major research in *Z. tritici* has focused on bread wheat. The genetics of STB-resistance in durum wheat remains poorly understood. Only a few studies on the inheritance of STB in durum wheat have been reported (Ferjaoui *et al.*, 2011; Berraies *et al.*, 2013a). The study of inheritance of STB resistance in durum wheat through a cross made between the resistant cv. "Salim" and the susceptible "Karim" showed that the resistance was quantitative, controlled by several genes with minor effects (Berraies *et al.*, 2013a). In search of sources of resistance in landrace cultivars, Ferjaoui *et al.* (2011) developed a mapping population obtained from a cross between the Tunisian landrace "Agili" that showed a good level of resistance and the susceptible cv. "Karim" using the isolate Tun06. The results showed the segregation of a single major gene. This gene was associated with AFLP markers but has not yet been assigned to a chromosome (Medini *et al.*, 2014).

1.11.10Phenotyping of STB disease

The study of STB resistance has been undertaken on different levels considering plant age (seedling, adult), plant structure (single leaf, single plant, and canopy) and plant environment (petri dish, growth chamber, greenhouse, and field). Disease screening can be done either in controlled conditions using a single isolate or an isolates mixture with a known virulence reaction or in field conditions. In field conditions, STB disease can be screened under natural

disease infection as well as after artificial inoculation. Different scoring scales are used in seedling and adult stages.

1.11.10.1 Seedling stage phenotyping

Screening at the seedling stage in a glasshouse as compared to screening adult plants and field trials means more genotypes can be scored in a smaller area in a shorter time period with no vernalisation requirement, giving quick results. To ensure a sufficient infection process, seedlings have to be kept in the dark at near 100% humidity for 48 hours after inoculation. Afterwards a day/night rhythm with a moderate light intensity for 16 hours, a temperature regime of 18/22 °C and a humidity of 70% is utilized. Usually the first leaves are inoculated and STB symptoms are scored 21 day post inoculation (dpi) (Kema *et al.,* 1996a). Several scores over time are taken to help calculate the area under the disease progress curve (AUDPC) (Chartrain *et al.,* 2004; 2005b) that gives an idea about disease progress over time. As the pathogen infection causes leaf necrosis and apparition of pycnidia that are responsible for disease dispersion, both parameters are scored as leaf area covered with necrosis and necrotic leaf area bearing pycnidia respectively on a quantitative scale. However, in some studies only pycnidial coverage (Chartrain *et al.,* 2005a; Arraiano *et al.,* 2007; Ghaffary *et al.,* 2012) or necrotic leaf area (Simon *et al.,* 2001) was scored.

Detached seedling leaf assays were developed for the wheat-*Z. tritici* pathosystem (Arraiano *et al.,* 2001a). This technique has the potential of testing several leaf sections of different genotypes at the same time in a petri-dishes with water agar media. After leaf inoculation with a specific isolate, the petri-plates are kept in the growth chamber at 20°C with 16 hours photoperiod and can be scored several times for the percentage of the necrotic blotches on which the typical black pycnidia are formed (Kema *et al.,* 1996a; Arraiano *et al.,* 2001a).

1.11.10.2 Adult stage phenotyping

The adult plant tests are needed because specificity of resistance to plant developmental stages has been observed (Kema *et al.,* 1996c; Ghaffary *et al.,* 2012). Adult plant resistance was studied either on single plants in the greenhouse (Adhikari *et al.,* 2004a; Simon *et al.,* 2001) or in the field (Simon *et*

al., 2004b). Under field conditions, wheat can be screened either under natural infection of the fungal population (Arraiano *et al.,* 2009) or after inoculation with mixtures of diverse *Z. tritici* isolates, more or less adapted to a specific environment (Jackson *et al.,* 2000). Field inoculation can be done before heading, one to three times at different growing stages or once after the full emergence of the flag leaf. Inoculation must be done either late in the afternoon to avoid high temperatures, when it's sunny with additional sprinkler irrigation (Ghaffary, 2011) or on a cloudy day with high humidity (Risser *et al.,* 2011) to ensure the infection.

STB disease severity in the field is best evaluated using the double-digit scale (00–99) developed as a modification of Saari and Prescott's scale for assessing the severity of foliar diseases in wheat (Saari and Prescott, 1975; Eyal *et al.,* 1987). The first digit (D1) indicates disease progress in the canopy height from the ground level; the second digit (D2) refers to measured severity based on infected leaf area. Both D1 and D2 are scored on a scale of 1 to 9. As the rate of disease progress in the field can be extremely fast in some regions conducive environmental conditions, it is often necessary to take repeated scores to properly assess the level of resistance (Dubin *et al.,* 1998). It is recommended that several individual disease scores should be taken at 3- to 7-day intervals over a 3- to 4-week period between anthesis and the dough stage. For each score, percentage disease severity is estimated and the AUDPC can be calculated using the percentage severity estimates corresponding to the three to four ratings which provides an idea about the rate of progress of the disease over time according to the plant stage. Some reported field experiments assessed only disease severity of STB symptoms on the flag leaf (STB lesions) (Ghaffary *et al.,* 2012; Eriksen *et al.,* 2003).

More difficult is the separation of disease escape and STB resistance in field experiments with natural inoculation of the locally occurring *Z. tritici* population. Disease escape occurs when a susceptible host does not become infected even under favourable environmental conditions. Both plant height and heading date may influence STB resistance (Somasco *et al.,* 1996; Arama *et al.,* 1999; Simon *et al.,* 2005) and must therefore be considered in disease escape. The taller plants tend to be more resistant because higher leaf levels have to be reached by the fungus. Earlier cultivars tend to be more affected by STB

because the flag leaf, which accounts for the main assimilate production, is affected for a longer time period than later heading cultivars (Shaw and Royle, 1993). The advantage of artificial field inoculation is that disease escape such as plant height and heading date can be avoided. Arraiano *et al.* (2009) observed the contribution of the disease escape traits such as plant height, leaf spacing, leaf morphology and heading date to STB resistance on more than 200 wheat lines under natural infection in the field. The authors supported a positive correlation between plant height and STB resistance previously identified in several studies (Simon *et al.*, 2004; 2005).

1.11.11Breeding for STB disease resistance

Breeding for STB resistance is the most effective, economical, and environmentally safe strategy to control this wheat disease. Several sources of resistance have been used to develop mapping populations to identify potential resistance genes. Even though 20 major resistant genes have been identified, as well as 167 QTLs in a total of nineteen bi-parental mapping populations, as described above (Brown *et al.*, 2015), this number is still considered as a limited arsenal compared to the number of genes identified for foliar wheat diseases such as yellow rust, leaf rust and powdery mildew, where the number of genes identified has so far reached 88, 96 and 104 respectively (Ghaffary *et al.*, 2011; Komugi, 2011).

So far two *Stb* genes (*Stb1* and *Stb4)* were reported to be incorporated into wheat varieties by breeders in several wheat improvement programs. *Stb1* was incorporated into the Indian soft red winter cvs. “Oasis” and “Sullivan” by Patterson *et al*. (1975, 1979). This gene provided durable resistance to wheat in Indiana and other parts in the Midwestern United States. As with other diseases, breakdown of STB resistance genes has been observed. Breakdown of STB resistance by the *Z. tritici* pathogen is directly related to the active sexual reproduction of the pathogen which allows new genetic recombination enabling the pathogen to adapt to the new environment and host resistance factors. Some *Stb* genes have lasted longer than others, e.g. *Stb4* was bred initially into the cv. “Tadinia” and was effective for ∼15 years in California (Somasco *et al*., 1996) but lasted only two years in Oregon (Jackson *et al.*, 2000). *Stb1* was effective in Indiana for more than 25 years. *Stb6* is known to be the most wildly spread *Stb*

gene in European cultivars (Arraiano and Brown, 2016). However, some varieties carrying *Stb6* were described as susceptible (Chartrain *et al.*, 2005a). It has also been shown that some *Stb* genes effective in one region can be non-effective in another (Makhdoomi *et al*., 2015). A study on the effectiveness of *Stb* genes to 116 Iranian isolates showed that among all the *Stb* genes, only *Stb15, Stb16q* and *Stb17* were effective and conferred resistance against all the isolates tested (Makhdoomi *et al*., 2015). Such examples emphasize that utilization of qualitative resistance through deployment of major gene resistance can be successful, but also has the potential for catastrophic failure when the pathogen overcomes the resistance gene (Hovmoller *et al*., 2008). Thus, breeding strategies for STB disease should focus on the combination of qualitative and quantitative resistance through pyramiding partial-resistance genes (Orton *et al*., 2011) as well as major resistance genes (Adillah *et al*., 2010). The application of this breeding strategy under field conditions and through testing under multiple environments would lead to the achievement of durable resistance (O'Driscoll *et al*., 2014; Raman and Milgate, 2012). Meanwhile, a continuous search for new sources of resistance is required to overcome the development of new virulence and to avoid the loss of effectiveness of resistant genes in cultivated wheat varieties.

1.12 Thesis outline and objectives

Durum wheat (*Triticum turgidum* L. ssp. durum) is the most commonly cultivated tetraploid wheat. Although durum wheat constitutes only 8% of world wheat production, it is an economically important crop because of its unique characteristics and end products such as pasta and semolina products (Ren *et al.*, 2013). As is the case with bread wheat, durum wheat faces multiple biotic and abiotic constraints with a direct impact on its yield. To face these challenges, breeders are in constant search to increase genetic diversity using different strategies as described above. One of these strategies is the production of pentaploid hybrids that consist of interspecific hybridisation between hexaploid and tetraploid wheat species. This strategy has the potential to improve the genetic background of either parent (Padmanaban *et al.*, 2017a) and help the transfer of a desirable trait(s) from either species to the other. In addition, Langdon D-genome disomic substitution lines (Joppa and Williams,

1977) can be used as a bridge in pentaploid crosses to facilitate the transfer of genes of interest located on the D-genome of bread wheat.

Moreover, the D-genome of wheat, as well as accessions of the D-genome progenitor *Ae. tauschii*, have been shown to be an important source of desirable genes for wheat improvement such as genes for abiotic stress tolerance, quality related traits and disease resistance. However the D-genome has been mainly used for bread wheat improvement. Its use for durum wheat improvement has been very limited to a specific genomic regions carrying genes of interest such as quality related genes on 1D (Ceoloni *et al*., 2003, Lukaszewski, 2003) and 5D chromosomes (Morris *et al.*, 2011) and aluminium tolerance genes on 4D chromosome (Han *et al.,* 2014). This can be explained by the difficulty of D-genome introgression into durum wheat in the presence of the *Ph1* gene. Thus, the exploitation of other D-genomic regions and their introgression into durum wheat could be a possible method for durum wheat improvement by facilitating the introgression of a target genomic region on the D-genome in the future. In this context, the research presented in this PhD thesis aims at:

Chapter 3. The transfer of seven *Stb* resistance genes located on the A-genome (*Stb6*, *Stb7*, *Stb12* and *Stb17*) and the D-genome (*Stb5*, *Stb10* and *Stb16)* of bread wheat into two susceptible durum wheat cultivars via pentaploid crosses. Subsequent generations were phenotyped in field conditions to *Z. tritici*-durum wheat specific isolates. Two different crossing strategies have been followed according to the position of the genes on either the A- or the D-genome. The potential transfer of the *Stb* genes in question in subsequent generations was assessed via their closely linked SSR markers. A GBS genotyping platform has been used for the characterisation of the genomic inheritance in pentaploid crosses from one side and the potential mapping of a genomic region of bread wheat that conferred resistance to STB disease in durum wheat from another side.

Chapter 4. The introgression of the D-genome of *Ae. tauschii* into durum wheat through the use of Langdon 5D (5B) substitution line to force translocations in the absence of the *Ph1* locus. The D-genome introgressions were characterised via GISH, FISH and D-genome SSR specific markers.

Chapter 5. The transfer of D-genome segments of bread wheat identified in wheat/*Am. muticum* introgression lines into two durum wheat genotypes. Hexaploid wheat/*Am. muticum* introgression lines were shown to carry inter-genomic rearrangements involving the D genome with either the A- or B-genomes, in the presence and absence of segments of *Am. muticum*, were crossed and back-crossed to two durum wheat genotypes. The D-genome segments were tracked, while advancing generations, via GISH until they reached a homozygous state in a tetraploid background (2n=28).

2 Chapter II. Materials and methods

2.1 Plant material

2.1.1 Seed sterilisation and germination

Seeds were sterilised with 1ml of 5% sodium hypochlorite (ClNaO) in a sterile micro-centrifuge tubes (Appendix 1). Tubes were incubated for 15 minutes and shaken every 2 minutes. The seeds were then washed three times with sterile distilled water and transferred to sterile Petri-plates. The plates were kept at 20°C in the dark for seed germination.

2.1.2 Vernalisation

Germinated seed were transferred into small pot trays at 25°C with a photoperiod of 12 hours. After five to six days, the seedling plants were transferred to vernalisation at 4°C and a photoperiod of 16 hours light and 8 hours dark cycle. The vernalisation period depended on the wheat type. Spring bread wheat and the durum wheat plants were vernalized for four and two weeks respectively. Wild relatives such as *Ae. tauschii* were vernalised for 8 weeks and backcrosses involving wild crosses from 4 to 8 weeks depending on the generation of the backcross.

2.1.3 Glasshouse conditions

At the end of the vernalisation period, plants were potted individually in 2 litre pots using John Innes No.2 compost in the glasshouse under an average temperature of 25°C and 16 hours photoperiod.

2.2 Wheat crossing method

2.2.1 Emasculation

Just after heading, the spikes of the female parent in pre-anthesis stage were emasculated: The central florets of each spikelet were removed. Using a pair of scissors, the top of the florets were cut off to allow easy access to the anthers. The three immature (green) anthers were delicately removed without disturbing the stigma, using forceps, from every single floret. The emasculated heads were then tagged (with the name of the plant and the emasculation date) and covered using a glassine bag for 1 to 3 days to prevent any accidental pollination until the stigma reached maturity. A mature stigma is 'fluffy'/feathered compared to an immature one which is tightly curled up.

2.2.2 Pollination

Spikes of the male parent that showed mature pollen were selected. Pollen maturity is reached when the anthers start to turn yellow, slightly rounded and open at the top. Having different batches of pollen plants increased chances of having mature pollen when the stigma is receptive. Before starting the pollination, the emasculated female heads are checked in case any anthers were accidently forgotten and released pollen. In this case the head was discarded. When the emasculated head was ready to be pollinated, the mature anthers were carefully removed from the floret of the pollen donor and gently taped inside the floret to drop the pollen on the receptive stigmas. Once the whole spike had been pollinated, the date of pollination on the crossing tag was recorded with the name of the donor and pollination date and the ear was bagged with the tag inside and closed with a tie.

2.3 DNA extraction

Leaf tissue was collected into 2ml micro-centrifuge tubes from the youngest leaves. After the plant material was freeze-dried for 16 hours, a steel ball was added to each tube and the samples were ground to a powder using a tissue lyser (QIAGEN; TissueLyser II) at a power of 25 Hz/second for 6 mins. 600 µl of extraction buffer solution (Appendix 2) was added and the samples incubated at 65^{o}C for 1 h after thorough mixing by shaking. The tubes were then placed on ice for 10 mins. 400 µl of ammonium acetate (chilled at 4^{o}C; Appendix 3) was added to the samples and kept on ice for 15 mins. The samples were then

spun down and the supernatant mixed with 400 µl of phenol/chloroform (1:1, V/V). The tubes were mixed gently and then centrifuged for 5 mins at 13000 rpm. 300 µl of isopropanol was added to the supernatant for DNA precipitation and kept on ice (4ºC) for 15 mins. The tubes were spun for 5 mins at 13000 rpm and the supernatant discarded. The pellet of DNA was re-suspended in 400 µl of 70% ethanol to wash and re-spun for 3 mins at 13000 rpm. The pellet was air dried at 37ºC for 5-10 mins and re-suspended in 100 µl of H_2O. The DNA was mixed by gently tapping and was then stored at -20ºC.

2.4 DNA quantification

The concentration of DNA was checked using either a Qubit 2.0 fluorometer (InvitrogenTM) or a Nanodrop 2000 spectrophotometer (Thermo Scientific), and estimated in ng/µl. Dilutions were then made using the numerical formula below:

$$\mathbf{C_i \times V_i = C_f \times V_f}$$

Where C is the concentration and V is the volume. The "i*"* and "f" indices are the initial and final values respectively.

2.5 Genomic *in situ* hybridization (GISH) and Fluorescence *in situ* hybridisation (FISH)

The protocol for GISH and FISH was as described in King *et al.,* (2017) and Grewal *et al.,* (2018) respectively.

2.5.1 Preparation of chromosomes spreads

Two roots per germinated seed were excised (1 cm) and treated with nitrous oxide gas at 10 bar for 2 h. Treated roots were fixed in 90% acetic acid for 10 mins and then washed three times with water, on ice. The root tip section containing dividing cells (1-2 mm) was dissected and digested in 20 µl of 1% pectolyase Y23 and 2% cellulase Onozuka R-10 (Yakult Pharmaceutical, Tokyo) solution (Appendix 4) for 50 mins at 37°C. After digestion, the root tips were washed three times in 70% ethanol. The root tips were carefully crushed in 100 µl of 70% ethanol using a needle. The cells were collected by centrifugation at 5000 rpm for 2 minutes, briefly dried and gently re-suspended in 20-35 µl of 100% acetic acid before being placed on ice. The cell suspension was dropped onto glass slides (7 µl per slide) in a moist box and dried slowly under cover.

2.5.2 Multi-colour GISH probe preparation

Genomic DNAs from young leaves of the three putative diploid progenitors of bread wheat, i.e. *T. urartu* (A-genome), *Ae. speltoides* (B-genome) and *Ae. tauschii* (D-genome), were extracted using the same protocol as described above. The genomic DNA of *Ae. speltoides* was fragmented to 300-500bp using a heat block for 15 minutes at 110°C. The genomic DNA of *T. urartu* was labelled by nick translation with Chroma Tide Alexa Fluor 488-5-dUTP (Invitrogen; C11397). Genomic DNA of *Ae. tauschii* was labelled with Alexa Fluor 594-5-dUTP (Invitrogen; C11400). Reaction components (Appendix 5) were prepared in a volume of 20µl to make the probes, then incubated at 16°C for two hours in the dark.

2.5.3 Multi-colour FISH probe preparation

For multi-colour fluorescence *in situ* hybridization (mc-FISH), two repetitive DNA sequences pSc119.2 (McIntyre *et al.,* 1990), and pAs.1 (Rayburn and Gill 1986) were labelled by nick translation with Chroma Tide Alexa Fluor 488-5-dUTP (green) and Alexa Fluor 594-5-dUTP (red), respectively. The reaction components (Appendix 6) were prepared in a final volume of 20 µl and then incubated at 16°C for two hours in the dark.

2.5.4 Probe precipitation

For probe precipitation, 160 µl of single stranded DNA (SS DNA) working solution (Appendix 7) was added to the 20 µl nick translation probe reaction and vortexed. 500 µl of 3M sodium acetate solution (Appendix 8) was added to the reaction mixture before being incubated at -20°C overnight. The tubes were centrifuged at 12,000 rpm for 30 mins at 4°C and the pellets washed with 70% ethanol and centrifuged for 5 mins (at 12,000 rpm) twice .After each spin the supernatant was discarded. After air-drying the pellets in the dark for 5-10 mins, they were dissolved in 20 µl of 2x SSC+1xTE buffer (Appendix 9) and stored at -20°C in the dark.

2.5.5 Probing the slides

The slides with the chromosome preparations were placed in the UV Cross link at 0.125 Joules twice. The probe mix for sc-GISH (Appendix 10) mc-GISH (Appendix 11) or mc-FISH (Appendix 12) was then added to the slide and covered with a plastic cover slip. For mc-GISH probing, slides were labelled

with *T. urartu* and *Ae. tauschii* probes and fragmented DNA of *Ae. speltoides* as blocker to detect the AABBDD genomes of wheat. For mc-FISH the prepared probes pSc119.2 and pAs.1 were used. The slides were placed on wet tissue paper in a stainless-steel tray and covered to keep them in the dark. The tray was placed in a just boiled container of deionized water (heat source turned off) for 5 mins. The denatured slides were transferred to a container with wet tissue and closed with a lid and incubated at 55°C overnight (minimum 12 hours). The plastic cover slips were removed by dipping the slides into 2× SSC (2×Saline-sodium citrate) solution (Appendix 9). A drop of Vectashield mounting medium with DAPI (4, 6-diamidino-2-phenylindole) or 1:2 diluted with 1×PBS (1×phosphate buffered saline) were added on the top of the slide and covered by a 24x50mm glass cover slip. All slides were counterstained with DAPI and analysed using a high throughput, fully automated Zeiss Axio Imager.Z2 upright epifluorescence microscope (Carl Zeiss Ltd, Oberkochen, Germany) with filters for DAPI (blue), Alexa Fluor 488 (green) and Alexa Fluor 594 (red). Photographs were taken using a MetaSystems Coolcube 1 m CCD camera. Further slide analysis was carried out using Metafer (automated metaphase image capture) and ISIS (image processing) software (Metasystems GmbH, Altlussheim, Germany).

2.6 Simple sequence repeat (SSR) marker

2.6.1 PCR reaction

Polymerase chain reaction (PCR) was used to amplify SSR markers in 96 well plates of 0.5 ml. Typical PCR reaction contained 2 µl of DNA template (50 ng/µl), 1 µl of 10×buffer with Mg^{++} (25 mM magnesium chloride: MgCl2), 0.2 µl of dNTP mix (5 mM dNTPs), 0.05 µl of taq polymerase (5U/ µl), and 0.5 µl of the forward and reverse primers at concentrations 1mM. Water was added to a final reaction volume of 10 µl.

2.6.2 PCR amplification condition

The PCR program used was: denatured at 94°C for 3 minutes, followed by 30 cycles with each cycle involving a 1 minute denaturation at 94°C, 30s primer annealing at 51-60°C depending on the marker and extension for 1 min at 72°C, with a final extension step of 72°C for 5 min. The final hold was at 10°C.

2.6.3 Visualisation of PCR product

Two µl of loading dye (6x DNA) was added to the 10 µl amplified product. The DNA was separated by gel electrophoresis using 3.5% superfine resolution agarose (Bioline, Molecular grade) gel. The gel was prepared in 1x TAE buffer (Appendix 13) and ethidium bromide was added at a concentration of 0.5 µg/µl. A 1Kb ladder was run along with the samples to estimate the product size. The gel was run at 110 Volts for 45 mins and visualised under UV light.

2.6.4 Data analysis

R-studio software (R-studio Team, 2015) was used for correlation analysis. The Pearson correlation test was used to assess the correlation between Septoria disease severity (SDS) and plant-height. The assessment of the effect of the *Stb* genes on disease resistance in durum wheat was calculated using the chi-square test of independence.

3 Chapter III. The use of Pentaploid crosses for durum wheat improvement to Septoria tritici blotch

3.1 Introduction

Breeding cereals, including durum wheat, with improved yield and quality performance has become an urgent and relevant need to ensure food security. Biotic stresses, caused by many diseases and pests have a major role in reducing wheat yield and quality in most wheat-growing areas worldwide. Septoria tritici blotch (STB), caused by *Zymoseptoria tritici* is considered the most common and damaging pathogen of wheat globally (O'Driscoll *et al.,* 2014; Gurr and Fones, 2015). Resistance to STB disease has been a significant target trait in wheat breeding since the sixties. The potential threat posed by this disease was drawn to international attention by a very damaging epidemic in North Africa in 1968-1969 (Saari and Wilcoxson, 1974). Subsequently, STB increased in importance, particularly in semi-dwarf cultivars given the high rates of nitrogen fertiliser (Wiese, 1987). Epidemics are most severe in areas with extended periods of cool, wet weather, particularly in North and South America and East Africa. However, most damage occurs in Europe and in the Central and West Asia and North Africa regions (Goodwin, 2012).

Given the importance of wheat globally and locally, and considering the potential losses due to STB, adequate control of STB is an urgent need. Currently, to limit crop losses, major control management strategies consist of fungicide applications. However, it's a short-lived strategy due to the rapid development of fungicide resistant strains of *Z. tritici*. Resistance to strobilurin and triazole fungicides (Boukef *et al.*, 2012; Cools and Fraaije, 2012; Drabešová *et al*., 2012; Torriani *et al.,* 2015 Torriani *et al.,* 2015), as well as insensitivity to succinate dehydrogenase inhibitors (SDHIs) (Teagasc, 2015) have already been reported. Thus, STB resistance has become one of the highest priorities in wheat breeding (Brown *et al.,* 2015; Torriani *et al.,* 2015).

Host plant resistance is considered to be the best strategy of STB management. The use of resistant cultivars reduces the need to apply fungicide treatments and provides a more reliable strategy for disease control. To date, resistance in durum wheat has not been well addressed; hence in countries where durum wheat is cultivated, important yield losses are recorded following STB recurrent infections. STB is currently one of the major problems of durum wheat in North Africa and especially Tunisia, where under conducive conditions, losses often exceed 60% (Fakhfakh *et al.,* 2011; Ferjaoui *et al.,* 2015). Thus, the need to breed for durum wheat resistance to STB disease to be able to defeat this pathogen and enhance the yield potential in this region.

3.1.1 The use of pentaploid crosses in wheat breeding

3.1.1.1 Pentaploid hybrid

Interspecific hybridisation between hexaploid and tetraploid wheat species leads to the development of F_1 pentaploid hybrids with unique chromosomal constitutions that can improve the genetic background of either parent by transferring traits of interest. Hybridisation between these two species with different ploidy levels lead to a pentaploid hybrid (AABBD) that has the chromosomal constitution of 2n=5x=35 (Kihara, 1924). Genetic variability that is combined from hexaploid and tetraploid wheat into a pentaploid hybrid has great potential in crop improvement (Eberhard *et al.,* 2010; Martin *et al.,* 2013; Kalous *et al.,* 2015). For production of interspecific wheat hybrids, the higher ploidy-level species should be used as the maternal parent because this will result in a greater number of fertile F_1 progeny (Kihara, 1982). Crosses in which

the lower ploidy level species has been used as the female have generally been less successful and can lead to poor seed set and subsequent low levels of seed germination and seedling establishment (Sharma and Gill, 1983; Padmanaban *et al.,* 2017a, 2017b). Recently, there has been renewed interest in the use of these pentaploid crosses to improve elite bread and durum wheat lines for a number of economically desirable characters (Martin *et al.,* 2011, 2013; Han *et al.,* 2014, 2016; Kalous *et al.,* 2015). Even with complications of infertility of the F_1 seed produced by hybridisation between domesticated inter-ploidy species such as bread and durum wheat, many studies showed a successful transfer of useful genes. Pentaploid crosses could be a powerful breeding strategy that can be used to enhance wheat in either direction based on the parental choice.

3.1.1.2 Chromosome constitution and D-genome retention

Chromosome elimination is an essential process that takes place in subsequent generations derived from the F_1 pentaploid. It may take a few generations to resolve the complex process of chromosome pairing and to give rise to a stable durum or bread wheat line. Because of the pentaploid composition of this F_1 generation, subsequent generations undergo irregular chromosome pairing in the D genome. Thus, subsequent generations derived from F_1 pentaploid wheat hybrids can be broadly classified into three groups, based on the presence or absence of D-genome chromosomes. The progeny belonging to the first group have lost all seven D-genome chromosomes (2n=4x=28); the second group consists of progeny that have intermediate numbers of D-genome chromosomes (total chromosome numbers ranging from 2n=29 to 41); while the third group have retained two copies of all seven D-genome chromosomes (2n=6x=42) (Padmanaban *et al.,* 2017b). Based on the objective of the breeding program that aims to develop bread or durum wheat lines, these three groups of pentaploid-derived wheat hybrids can be selfed or backcrossed with either parent. Significant differences in the retention of D chromosomes in the F_2 generation depending on the parents of the original cross were observed in hexaploid/tetraploid crosses (Padmanaban *et al.,* 2018). Cytological analysis at the F_5 generation showed that D-genome chromosomes were still being eliminated, suggesting that in some hexaploid/tetraploid

crosses, D-genome chromosomes can be unstable for many generations (Padmanaban *et al.,* 2018). Martin *et al.,* (2011) reported that particular parental combinations played a key role in determining the retention of D chromosomes in the successive generations of hexaploid/tetraploid wheat crosses. There also appears to be a strong correlation between the retention of D-genome chromosomes and the inheritance of the A- and B-genomes. Lines that retained a large number of D-genome chromosomes also inherited a higher proportion of the A- and B-genomes from bread wheat (Martin *et al.,* 2011; Padmanaban *et al.,* 2017b)

3.1.1.3 Application in wheat breeding

Several studies have shown the potential of pentaploid crosses to transfer genes of interest for biotic or abiotic stresses from bread wheat into durum wheat or the other way-round (Munns *et al.,* 1999; Lopes *et al.,* 2010; Han *et al.,* 2016). Genes for disease resistance have been successfully transferred into durum wheat using bread wheat as a source of resistance. For example, resistance to crown rot disease in durum and related tetraploid wheat species has not been identified. However, partial resistance to crown rot has been identified in some bread wheat genotypes, such as "Sunco", "2-49" and "CPI133814" (Wildermuth *et al.,* 2001; Bovill *et al.,* 2006, 2010). This partial resistance in the hexaploid source is associated with multiple chromosomal regions, including 1A, 1B, 1D, 3B, and 4B in "2-49", and 2B in "Sunco" (Bovill *et al.,* 2010; Martin *et al.,* 2015). Subsequent hexaploid/tetraploid crosses were made using these partially resistant sources "2-49" and "Sunco" with susceptible durum line "950329". Based on the complete absence of D-genome chromosomes and crown rot resistance, F_6 lines were selected and backcrossed with the durum parent. The BC_2-F_2 progeny were assessed for crown rot severity in field conditions. Lines with a better level of resistance than the hexaploid "2-49" genotype were identified (Martin *et al.,* 2013). This result suggests that crown root resistance can be successfully introgressed into durum wheat from the bread wheat source. FHB is another successful example of resistance transfer from bread wheat into durum wheat. The Chinese bread wheat variety "Sumai 3" and the two bread wheat cultivars "Ning8331" and "93FHB21" have been identified as resistant sources for FHB. In an attempt to transfer this

resistance into durum wheat, crosses were made with the susceptible durum wheat cultivars “Stewart 63” and “DT486”. The resulting F_1 pentaploid hybrids showed improved resistance to FHB compared to the susceptible durum parent (Gilbert *et al.,* 2000). The majority of F_2 plants from the two pentaploid crosses with “Sumai 3” had the visual appearance and level of resistance of “Sumai 3”. Using D-genome specific SSR markers, no relationship between the presence/absence of D-genome chromosomes has been associated with the FHB reaction in the F_2 plants (Gilbert *et al.,* 2000).

Other studies showed that genes of resistance were successfully introgressed from durum wheat into bread wheat. The dominant resistance gene *Yr53,* present in the Ethiopian durum wheat accession “PI 480148” conferring yellow rust resistance, was successfully transferred into the susceptible bread wheat cultivar “Avocet” through pentaploid crossing (Xu *et al.,* 2013). In fact, the progeny derived from the crosses, were cytologically selected based on the presence of all seven pairs of D chromosomes and tested with stripe rust race PST100. The progeny of the F_3 generation segregated in a 3:1 resistant to susceptible ratio, suggesting that a single dominant gene was responsible for the resistance. In addition, pentaploid hybrids, derived from crosses of the powdery mildew (caused by the fungus *Blumeria graminis f. sp. Tritici*) susceptible hexaploid wheat cultivars “Maris Nimrod” and “Norman” with resistant tetraploid *T. dicoccoides* accession CLI060025, showed improved resistance to powdery mildew when the stable F_3 progeny were back- and top-crossed to a second hexaploid wheat (Reader and Miller, 1991).

In a study aiming to investigate the impact of durum alleles in a hexaploid background and bread wheat alleles in a tetraploid background for important quantitative traits, and to evaluate the impact of the D-genome on several agronomic and quality traits, an F_5 RIL populations at both hexaploid and tetraploid levels were developed from a cross between the hexaploid spring wheat “Choteau” and the tetraploid durum wheat “Mountrail” (Kalous *et al*., 2015). The tetraploid and hexaploid F_5 plants were assayed using specific markers for each of the D-genome chromosomes. These lines, however, contained a mixture of alleles from the A- and the B-genomes. Several QTLs for agronomic and quality traits were identified in both the 4X and 6X populations. Results showed that the D genome had a major impact on most

yield and yield-related traits measured in the F_5 RIL populations, e.g. presence of the D genome resulted in greater tiller number in the hexaploid plants. Although negatively impacted by the lack of the D genome, kernel weight in the tetraploid plants was higher than the hexaploid due to positive alleles from the durum cultivar "Mountrail" on chromosomes 3B and 7A (Kalous *et al*., 2015).

3.1.1.4 Genomic translocation in pentaploid hybrid derived line

If suitable genes are located on either the A or B genomes of hexaploid wheat or related diploid species, they can be easily introgressed into durum wheat without the need for the *Ph1* mutation. The interspecific crosses have potential to improve modern bread and durum wheat cultivars. The transfer of genes of interest, located on the D genome of bread wheat, into durum wheat through conventional breeding can be very challenging considering the presence of the *Ph1* gene. In advanced generations of pentaploid crosses, D-genome chromosomes are predominantly inherited as complete chromosomes. However, spontaneous translocations between the D-genome and the A- or the B-genomes may occur in pentaploid derived hybrid lines, but at low frequency (Eberhard *et al.*, 2010; Martin *et al.*, 2011). There is a tendency for the introduced univalent chromosomes to undergo centric breakage-fusion leading to chromosomal translocations/deletions (Sharma and Gill, 1983). Screening of 26 F_2 progeny of a pentaploid cross, mc-FISH analysis showed that all plants contained varying amounts of D genome material. However, five (19%) carried A-D translocations, of which two were telocentric and three telomeric translocations (Padmanaban *et al.*, 2017a).

3.1.2 Langdon D-genome disomic substitution lines

Langdon D-genome disomic substitution lines (LDN-DS) developed by Joppa and Williams in 1988, are a set of 14 lines in which a pair of Chinese Spring (CS) D-genome chromosomes substitute a corresponding homoeologous pair of A- or B- genome chromosome of LDN. The LDN-DS lines were derived from initial crosses of the common wheat cultivar 'Chinese Spring' (CS) nulli-tetrasomics, which were nullisomic for an A- or B-genome chromosome and tetrasomic for a corresponding homoeologous D-genome chromosome, with LDN (Joppa and Williams, 1988). Double monosomics for an A- or B-genome

chromosome and a corresponding homoeologous D-genome chromosome selected from these crosses were originally backcrossed six times with LDN to develop the LDN-DS lines (Joppa and Williams, 1988). A set of 14 LDN-DS lines have subsequently been backcrossed with LDN for five additional backcrosses to reduce the residual background. Nine of the LDN-DS lines produced this way contain 13 pairs of A- and B-genome chromosomes and 1 pair of D-genome chromosomes. The other 5 LDN-DS lines; 4D (4A), 5D (5A), 5D (5B), 3D (3B), and 6D (6B), however, were maintained in the presence of the chromosome or an arm of the chromosome substituted by the D-genome chromosome. Therefore, LDN-DS lines 4D (4A), 5D (5A), 5D (5B), 3D (3B), and 6D (6B) contain a monosome or telosome (4A, 5A, 5B, 3BL, and 6BS, respectively), in addition to the other 13 pairs of A- and B-genome chromosomes and one pair of D-genome chromosomes (Joppa and Williams, 1988).

The LDN-DS lines have been widely used in genetic and genomic studies of tetraploid wheat. These lines were mostly exploited for genes localisation in tetraploid wheats (Konzak and Joppa, 1988; Joppa and Cantrell, 1990; Cantrell and Joppa, 1991; Tsunewaki, 1992; Cai *et al.*, 1999; Shimelis *et al.*, 2005) on the basis of alterations in phenotypic value, when the durum chromosome is substituted. In addition, LDN-DS lines have been of a great help to transfer genes located on the D-genome into a durum wheat background. For instance, the *TaALMT1* allele, associated with Al^{3+} tolerance, located on the chromosome 4D has been transferred into durum wheat to increase its aluminium tolerance, especially for durum wheats growing on acid soil (Han *et al.*, 2014). In fact, the LND-DS line 4D (4B) was crossed to the *Ph1* mutant cv. Cappelli (*ph1c*) and a chromosomal segment of 4D was successfully introgressed into durum wheat, which substantially enhanced Al^{3+} tolerance in the sister lines derived from three generations of backcrossing (Han *et al.*, 2014).

3.1.3 The aims of the chapter

To the best of our knowledge none of the seven *Stb* genes have been studied in durum wheat background. Pentaploid crosses between bread and durum wheat have been shown to be a potential breeding strategy to enhance either of the species depending on the direction of the crosses. This strategy can be used

for *Stb* gene transfer into durum wheat. Considering the pathogenic dimorphism of *Z. tritici* isolates toward wheat species, these crosses will help study/identify the potential genomic region(s) responsible for bread wheat resistance to durum wheat isolates and its transfer into durum background.

The hypotheses of this chapter are:

1. The ability of the *Stb* genes located in the bread wheat to confer resistance to STB disease when transferred into durum wheat.
2. The feasibility of the transfer the *Stb* genes located on the D-genome into durum wheat background using the Langdon D-genome substitution.
3. To study of the genomic inheritance of the A- and the B-genomes of bread wheat and the D-genome retention in advanced generations of pentaploid populations backcrossed to durum wheat.
4. The feasibility of the bread wheat to confer resistance to durum wheat for STB disease test via QTL analysis of one population "C1" excluding the D-genome markers and the effect of plant height on disease resistance.

3.2 Material and methods

3.2.1 First crossing strategy: Direct bread wheat/durum wheat crosses

3.2.1.1 Plant material

The first crossing strategy consisted of a direct cross of five hexaploid genotypes resistant to STB disease, known to carry specific *Stb* genes on the A- and the D-genome (**Table 3-1**), to two susceptible tetraploid wheat (*Triticum turgidum*) genotypes "Karim" and "Om Rabiaa 5" known to have a highly and moderately susceptible reaction to STB disease, respectively. Two of these bread wheat genotypes "Balance and Tadina" are a winter wheat type that require eight weeks of vernalisation, whereas the rest of the bread wheat genotype are spring type and require only four weeks of vernalisation.

Table 3-1. *Stb* resistant gene in three hexaploid wheat cultivar and their chromosomal location.

Hexaploid wheat genotypes	*Stb* gene on the A- genome (locus)	*Stb* genes on the D-genome (locus)
Synthetic 6X	*Stb6* (3AS)	*Stb*5 (7DS)
Kavkaz-K4500 (KK4500)	*Stb6* (3AS) *Stb7* (4AL) *Stb12* (4AL)	*Stb10* (1Dc)
SH M3	*Stb17* (5AL)	*Stb16* (3DL)
Balance	--	*Stb*18 (6DS)
Tadinia	*Stb6* (3AS)	*Stb*4 (7DS)

3.2.1.2 Crossing plan

Four plants of each hexaploid wheat genotype were planted at two weeks intervals on three different dates. Eight plants of both durum wheats "Karim" and "Om Rabiaa 5" were planted weekly to ensure pollen availability throughout the crossing season. After vernalisation, plants were potted in glasshouse conditions and every bread wheat genotype was crossed as the female parent to both of the durum wheat genotypes (as described in section 2.1). In total, ten cross-combinations were made (**Figure 3-1**). The F_1 pentaploid produced were then backcrossed twice to the recurrent durum wheat parent. Despite that F_1 seed was produced out of the crosses of the two durum wheat to the two winter type bread wheat "Balance and Tadina", the F_1 plants showed a hybrid necrosis symptoms and failed to reach maturity. Hence, these four cross-combinations were discarded and only six cross-combinations were kept as shown in **Figure 3.1.**

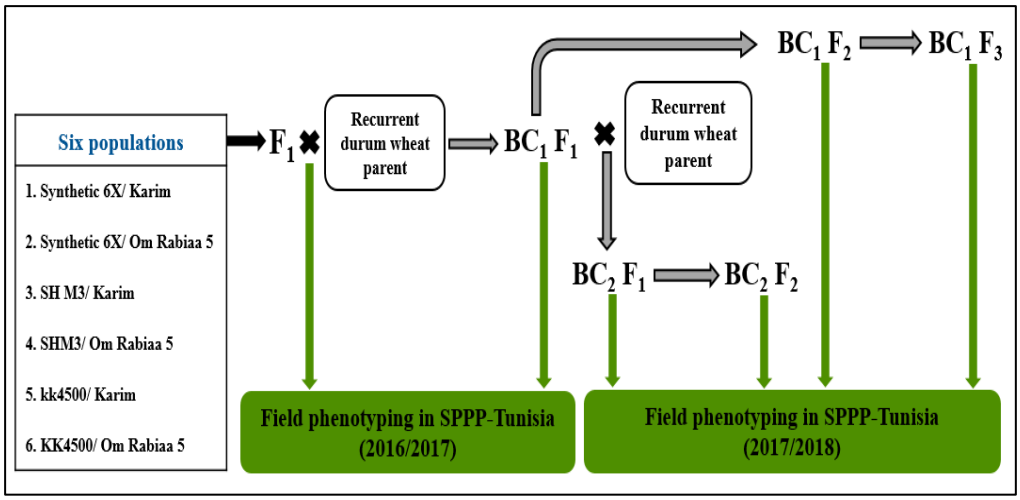

Figure 3-1. The first crossing strategy diagram: Direct bread wheat/durum wheat crosses.

3.2.2 Second crossing strategy: Three way crosses using LND-DS lines

3.2.2.1 Plant material

To transfer *Stb* genes located on the D-genome into durum wheat, six Langdon D-genome disomic substitution lines (LND-DS) were used in crosses with three resistant hexaploid wheat genotypes (**Table 3-1**). Each LND-DS line had a different pair of D-genome chromosomes that substituted either a pair of A- or B-genome chromosomes of the same homologous linkage group (e.g. 1D substituted for 1A or 1B). In the present study, the LND-DS lines were selected based on the presence of the D-genome chromosome of the same group as the D-genome chromosome harboring the *Stb* gene in the bread wheat genotype (**Table 3-2**).

Table 3-2. List of the Langdon D-genome disomic substitution lines used in the present study

LND-DS lines reference	Substitution	Hexaploid parent crossed with (*Stb* gene, location on D-genome)
LD-1 **LD-10**	1D (1A) 1D (1B)	KK4500 (*Stb10* on 1DC)
LD-4 **LD-11**	3D (3A) 3D (3B)	SH M3 (*Stb16* on 3DL)
LD-7 **LD-14**	7D (7A) 7D (7B)	Synthetic 6X (*Stb*5 on 7DS)

3.2.2.2 Crossing plan

F_1 hybrids were produced using bread wheat as the female parent and the selected LND-DS lines depending on the position of the *Stb* gene on the D-genome of the bread wheat, as the male parent (**Table 3-2**). For instance, the bread wheat cultivar SH M3, carrying *Stb16q* gene located on chromosome 3D was crossed to LD-4 and LD-11 lines carrying the 3D (3A) and 3D (3B) substitutions, respectively. Hence every bread wheat genotype was crossed to two LND-DS lines carrying the same pair of D-genome chromosomes substituting either the A- or the B-genome chromosomes (**Table 3-2**). In total, six cross-combinations were made. As represented in the crossing diagram below (**Figure 3-2**), the F_1 hybrids produced were then crossed and back-crossed to both of the durum wheat parents "Karim" and "Om Rabiaa 5" separately, generating 12 cross-combinations/populations of three way crosses noted in the rest of the manuscript as F_1T (F_1Top).

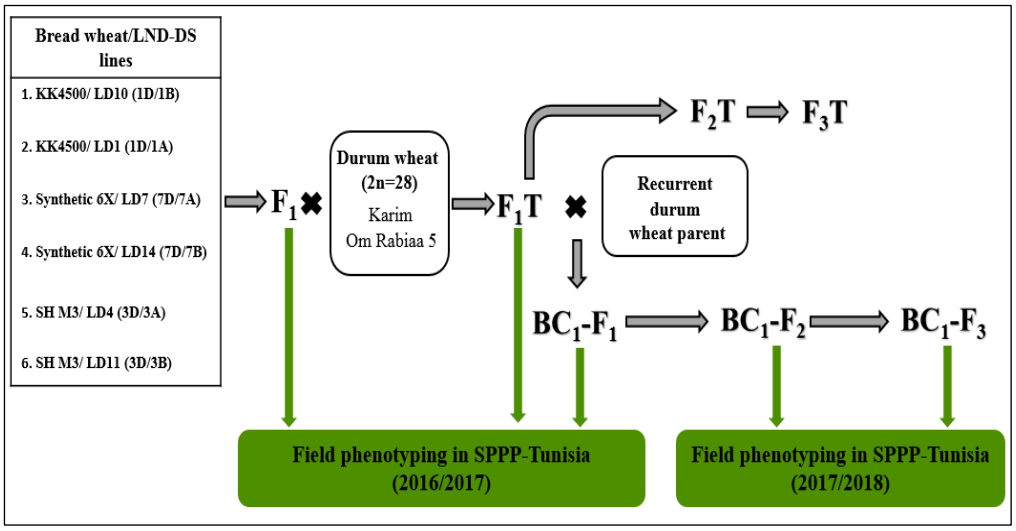

Figure 3-2. The second crossing strategy diagram: three way crosses using LND-DS lines.

3.2.3 Plant phenotyping under field condition for STB disease

3.2.3.1 Field experiment

The pentaploid F_1 seeds (35 chromosomes) as well as the advanced generations of all cross-combinations from the two crossing strategies (**Figure 3-1**, **Figure 3-2**) were screened for STB disease resistance under field conditions during two consecutive growing seasons 2016/2017 and 2017/2018

alongside the parental lines and the full set of LND-DS lines, in the Septoria Phenotyping Precision Platform (SPPP) based in Tunisia (**Table 3-3**).

Table 3-3. List of the full set of Langdon durum disomic D-substitution lines.

LND-DS line reference	Genomic disomic Substitution
LD- 1	1D (1A)
LD- 2	2D (2A)
LD- 3	2D (2B)
LD- 4	3D (3A)
LD- 5	4D (4A)
LD- 6	5D (5B)
LD- 7	7D (7A)
LD- 8	5D (5A)
LD- 9	6D (6A)
LD- 10	1D (1B)
LD- 11	3D (3B)
LD- 12	4D (4B)
LD- 13	6D (6B)
LD- 14	7D (7B)

Plants were sown in a complete randomised block design in the SPPP localized in northern Tunisia, known to be a natural hot spot for *Z. tritici* durum wheat specific isolates. The F_1 generation as well as the BC_1-F_1 from the first crossing strategy and the F_1T generation from the second crossing strategy were screened during the 2016/2017 growing season. The different lines (bulked seed of a single spike or crossed head) were hand sown in blocks of 100 rows of 1m long spaced by 25cm. Blocks were split into 2 sub-blocks each with 50 entries. The susceptible genotype "Karim" was used as a disease spreader in the field. Karim was sown in a continuous central line between every two blocks with 50cm equidistance (**Figure 3-3**).

During the 2017/2018 growing season, advanced generation of all the crosses (self-seed of selected plants from the previous growing season and seed of advanced generation in glasshouse conditions) were sown as described in **Figure 3-3**, with the only modification being row length (2m instead of 1m). For easy screening of individual plants for STB disease resistance, within the same row, seed were planted 15-20cm apart (rate of 60 plants /row).

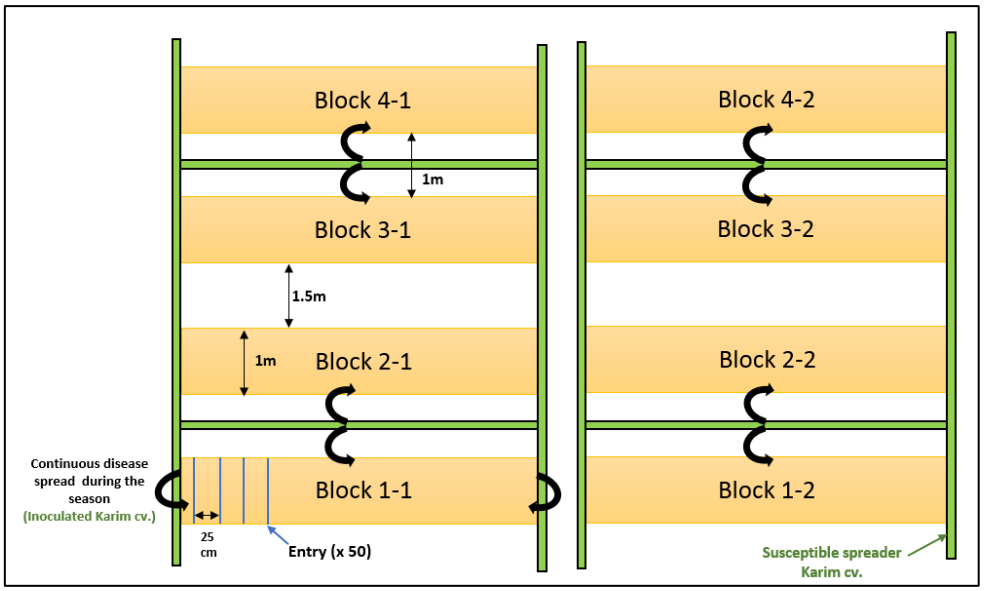

Figure 3-3. Field experiment design (green lines: inoculated Karim cv. used as disease spreader, black arrows; expected disease spreading direction).

3.2.3.2 Plant inoculation

To ensure a good level of infection of STB disease, the naturally infested field was also inoculated with infected straw applied straight after sowing to insure the primary infection. Three liquid inoculations were applied after the emergence of the fifth leaf and stem elongation growth stage, with two to three weeks interval between consecutive inoculations. The inoculum was prepared using a mixture of five aggressive isolates of *Z. tritici,* originating from the same region (north of Tunisia), at a concentration of 10^6 spore/ml applied directly on the plants and the susceptible Karim cv. used as a disease spreader (**Figure 3-3**). Liquid inoculations were prepared and applied by SPPP technicians with a high pressure motor sprayer. The local resistant variety "Salim" was used as a resistant check.

3.2.3.3 Disease assessment

Disease severity was visually scored straight after heading (in April) according to a double-digit scale (00-99) modified from Saari and Prescott (1975) for wheat foliar diseases. The first digit D1 represents the vertical disease progress as deriving from the average relative height reached by the disease from the ground level (0-9). The second digit (D2) represents the severity of the disease, measured as the average relative coverage of the diseased leaf area.

3.2.3.4 Evaluation of the STB infection

The percentage of disease severity was estimated using the following formula (used by Sharma and Duveiller, 2007):

$$\mathrm{SDS} = \left[\left(\frac{\mathrm{D1}}{9}\right)\left(\frac{\mathrm{D2}}{9}\right)\right]100$$

The SDS (Septoria disease severity) index is thus composed by a first digit representing the blotch development up the plant height (e.g. 5 if the disease reached the mid-point of the plant or 50%, 8 if it reached the flag leaf, 9 if it reached the spike), and a second digit representing severity (e.g. 1 for 10% to 9 for 90%). SDS values thus range from 0 to 100, where 0 would indicate complete resistance, and 100 would indicate complete susceptibility.

3.2.4 Screening of the LND-DS lines STB disease at seedling stage

3.2.4.1 Plant material

Two replicates of the full set of LND-DS lines (**Table 3-3**) alongside the "Langdon" genotype and the susceptible check "Karim" were screened for STB disease resistance at the seedling stage in controlled conditions (see below).

3.2.4.2 Pant inoculation

The inoculum was produced using a mixture of five aggressive isolates (the same as the isolates used in the field screening) with a concentration of 10^6 spore/ml applied 18 days after planting (i.e. two to three leaf stage) in the growth room using a manual hand spray. Straight after inoculation, plants were moved to a humid chamber with 100% humidity for 48 hours before being transferred back to the growth room at 20°C with 12 hours photoperiod.

3.2.4.3 Disease scoring

The STB disease infection was scored as the percentage of leaf area covered by necrotic lesions bearing pycnidia at 28 days post inoculations.

3.2.5 Cytogenetic analysis of the D-genome retention in the two crossing strategies

Randomly selected seed from the BC_1-F_2 and BC_2-F_1 generations for the pentaploid bread wheat/durum wheat populations (1^{st} crossing strategy) and from the F_2T and BC_1-F_1 generations for the 3 way-crosses involving the LND-DS lines (2^{nd} crossing strategy) (**Table 3-4**) were screened for D-genome

retention using a cytogenetic approach. Between four and eight seeds of all the populations were randomly selected. A total of 150 plants, depending on the number of seed obtained after plant-selfing or crossing (most of the seed were kept for field trial) were screened using mc-GISH (**Table 3-4**). Straight after germination, root tips were collected and the mc-GISH protocol followed as described in section 2.5.

Table 3-4. List of the plants analysed with mc-GISH.

Crossing strategy	Generation	Cross reference	Pedigree	No. of plants
First crossing strategy	BC_1-F_2	**C1**	KK4500/Karim*2	4
		C3	SH M3/Karim*2	8
		C4	SH M3/Om Rabiaa 5*2	8
		C7	Synthetic 6X/Karim*2	8
		C8	Synthetic 6X/Om Rabiaa 5*2	8
	BC_2-F_1	**C1**	KK4500/Karim*3	4
		C3	SH M3/Karim*3	4
		C4	SH M3/Om Rabiaa 5*3	6
		C7	Synthetic 6X/Karim*3	8
		C8	Synthetic 6X/Om Rabiaa 5*3	4
Second crossing strategy	BC_1-F_1	**C9-K**	SH M3/LD-11/2/Karim*2	4
		C9-O	SH M3/LD-11/2/Om Rabiaa5*2	4
		C10-K	SHM3/LD-4//Karim*2	4
		C11-K	Synthetic 6X/LD-7/2/Karim*2	4
		C11-O	Synthetic 6X/LD-7/2/Om Rabiaa5*2	4
		C14-K	KK4500/LD-1/2/Karim*2	4
		C14-O	KK4500/LD-1/2/Om Rabiaa 5*2	4
		C15-K	Synthetic 6X/LD-14/2/Karim*2	4
		C15-O	Synthetic 6X/LD-14/2/Om Rabiaa 5*2	4
		C18-K	KK4500/LD-10/2/Karim*2	4
		C18-O	KK4500/LD-10/2/Om Rabiaa 5*2	4
	F_2T	**C9-K**	SH M3/LD-11/2/Karim	4
		C9-O	SH M3/LD-11/2/Om Rabiaa 5	4
		C11-K	Synthetic 6X/LD-7/2/Karim	4
		C10-O	SH M3/LD-4/2/Om Rabiaa 5	2
		C11-O	Synthetic 6X/LD-7/2/Om Rabiaa 5	4
		C14-K	KK4500/LD-1/2/Karim	4
		C14-O	KK4500/LD-1/2/Om Rabiaa 5	4
		C15-K	Synthetic 6X/LD-14/2/Karim	6
		C15-O	Synthetic 6X/LD-14/2/Om Rabiaa 5	4
		C18-K	KK4500/LD-10/2/Karim	4
		C18-O	KK4500/LD-10/2/Om Rabiaa 5	4
Total number of plants				**150**

3.2.6 The transfer of *Stb* genes into durum wheat and their effect on STB disease resistance

3.2.6.1 Plant material

Six populations were generated from the first crossing strategy and 12 from the second crossing strategy, and thus the decision was made to select six populations in the most advanced stage of backcrossing to study the effect of *Stb* genes, transferred into durum wheat on disease resistance (this decision was made in consultation with Dr. Amor Yahyaoui, the director of the SPPPP). In total, 665 lines were screened alongside the parental lines (**Table 3-5**). Plants were assessed individually for STB disease resistance alongside the parental lines. Screening for STB disease consisted of using the double-digit scale (00–99) developed as a modification of Saari and Prescott's scale for assessing the severity of foliar diseases in wheat (Saari and Prescott, 1975; Eyal *et al.*, 1987). The first digit (D1) indicates disease progress in the canopy height from the ground level; the second digit (D2) refers to measured severity based on infected leaf area. Both D1 and D2 are scored on a scale of 1 to 9. Plant height was as well measured individually for all the plants.

Table 3-5. Pedigree and population size of the selected six populations.

Crossing strategy	Generation	Population reference	Pedigree	Population size
First crossing strategy	BC_1-F_2	C1	KK4500/Karim*2	99
		C7	Synthetic 6X/Om Rabiaa 5*2	138
	BC_1-F_3	C3	SH M3/Karim*2	150
		C4	SH M3/Om Rabiaa 5*2	119
Second crossing strategy	BC_1-F_2	C9	SH M3/LD-11 (3B/3D)//Karim*2	91
		C18	KK4500/LD-10(1D/1B)//Karim*2	68
Total number of plants				**665**

3.2.6.2 Molecular screening for the STB genes retention

Leaf material was sampled from all the screened plants (**Table 3-5**) and the parental lines. Freeze-dried leaf material of all samples were sent to CIMMYT, biotechnology laboratory (in collaboration with Dr. Susanne Dreisigacker) for

DNA extraction and screening for *Stb* gene retention using the corresponding SSR markers (as describe in sections 2.5 and 2.6).

3.2.7 Plant genotyping

DNA samples of four populations (C1, C3, C4 and C7) in the BC_1-F_2 and BC_1-F_3 generations described in **Table 3-5** were selected to study genomic inheritance in the pentaploid crosses (1st crossing strategy). Normalized DNA at 50 ng/µl was sent for DArTseq™ analysis in collaboration with Dr. Carolina Sansaloni. High-throughput genotyping was conducted in 96 plex using DArTseqTM technology (Sansaloni *et al.*, 2011) in the Genetic Analysis Service for Agriculture facility at CIMMYT, Mexico. A genomic representation of the samples was generated by digesting the genomic DNA with a combination of two restriction enzymes — PstI (CTGCAG) and HpaII (CCGG) — and ligating barcoded adapters to identify each sample to run within a single lane on the Illumina HiSeq2500 instrument (Illumina Inc., San Diego, CA). Successfully amplified fragments were sequenced up to 77 bases, generating ~500,000 unique reads per sample. A proprietary analytical pipeline developed by DArT P/L was used to generate SNP calls. A set of filtering parameters were then applied to select high-quality markers. To obtain the physical positions of the corresponding DArTseq markers, the sequences of the DNA fragments were BLASTed against a local database containing the wheat consensus map v.4 (diversityarrays.com) and to the wheat reference genome sequence from International Wheat Genome Sequencing Consortium (IWGSC) Refseqv1 (IWGSC, 2018), with expected values (E)<e10 and minimum base identity>90%.

3.2.8 Data analysis

Flapjack software was used to select and filter relevant polymorphic SNP markers in every population. Sequences that had missing values in more than 80% of the lines were removed. Marker assisted backcrossing statistical analysis included in the Flapjack software was used to calculate the recurrent parent allele's percentages for each line across each chromosome following an unweighted model (which doesn't take the amount of genome represented by each marker into consideration).

3.2.9 QTL's analysis

The C1 population in the BC_1-F_2 generation was analysed using the IciMapping V4.1 software for map contrasct and mapping of quantitative trait genes analysis. Inclusive composite of interval mapping of additive and dominant method was used for QTL mapping.

3.3 Results

3.3.1 Populations development

3.3.1.1 First crossing strategy: direct bread wheat/durum wheat crosses

In the attempt to transfer *Stb* genes localized on the A-genome from bread wheat into durum wheat, three bread wheats were crossed to two susceptible durum wheats which gave rise to six cross-combinations. A total of 73 crosses were made giving rise to 328 F_1 seeds (**Table 3-6**). A clear difference in the number of F_1 seed produced between the different combinations was observed. The bread wheat parent KK4500 gave the highest number of seed with the durum wheat parent Om Rabiaa 5 (C2). The same bread wheat parent gave the lowest number of seed when crossed with the durum wheat parent Karim, indicating the importance of the parental choice in the pentaploid crosses (**Table 3-6**).

Randomly selected F_1 plants were backcrossed to the recurrent durum wheat parent. The number of crosses in every population depended on the number of heads available and the availability of pollen and gave rise to 655 BC_1-F_1 seeds. Another round of back-crossing to the recurrent durum wheat parent was made to ten randomly selected BC_1-F_1 plants generating a total of 335 BC_2-F_1 seeds.

Table 3-6. Number of crosses and seed produced in glasshouse conditions of six pentaploid crosses in the F_1, the BC_1-F_1 and the BC_1-F_2 generations.

Pop. Ref.	F_1 Pedigree	F_1		BC_1-F_1		BC_2-F_1	
		No. of crosses	No. of seeds produced	No. of crosses	No. of seeds produced	No. of crosses	No. of seeds produced
C1	KK4500/Karim	11	46	6	35	5	73
C2	KK4500/Om Rabiaa 5	12	25	18	60	7	28
C3	SH M3/Karim	16	40	2	6	5	25
C4	SH M3/Om Rabiaa 5	8	52	6	49	4	37
C7	Synthetic 6X/Karim	14	85	39	290	13	125
C8	Synthetic 6X/Om Rabiaa 5	12	80	31	215	6	47
Total		**73**	**328**	**102**	**655**	**40**	**335**

One to two heads of the BC_1-F_1 plants were allowed to self-pollinate to produce the BC_1-F_2 seed. (**Table 3-7**). Crosses were advanced to the BC_1-F_3 generation. Seed from the C2 population failed to germinate and set BC_1-F_3 seed. However, 39 randomly selected BC_1-F_2 seeds of the other five populations produced a varied amount of BC_1-F_3 seed (**Table 3-7**).

Table 3-7. BC_1- F_2 and the BC_1-F_3 seed production in glasshouse conditions.

Generation		BC_1-F_2			BC_1-F_3	
Pop. Ref.	BC_1-F_1 Pedigree	No. of plants	Self-pollinated heads	No. of seeds produced	No. of plants selfed	No. of seeds produced
C1	KK4500/Karim*2	4	3	120	4	136
C2	KK4500/Om Rabiaa 5*2	9	4	27	-	-
C3	SH M3/Karim*2	6	11	235	14	1033
C4	SH M3/Om Rabiaa 5*2	4	7	177	8	461
C7	Synthetic 6X/Karim*2	7	8	249	6	536
C8	Synthetic 6X/Om Rabiaa 5*2	6	7	275	7	313
Total		**36**	**40**	**1083**	**39**	**2479**

The BC_2-F_1 plants were also advanced to the next generation (with the exception of the C2 family which was excluded from the study). Twenty eight randomly selected BC_2-F_1 plants were self-fertilised in glasshouse conditions generating 1934 BC_2-F_2 seed (**Table 3-7**).

Table 3-8. BC_2- F_2 seed production in glasshouse conditions.

Pop. Ref.	F_1 Pedigree	No. of plants selfed	No. of seeds produced
C1	KK4500/Karim*3	3	246
C2	KK4500/Om Rabiaa 5*3	-	-
C3	SH M3/Karim*3	3	232
C4	SH M3/Om Rabiaa 5*3	6	326
C7	Synthetic 6X/Karim*3	14	1063
C8	Synthetic 6X/Om Rabiaa 5*3	2	67
Total		**28**	**1934**

3.3.1.2 Second crossing strategy: Three way crosses using LND-DS lines

Six cross-combinations were made between the LND-DS lines holding a pair of D-genome chromosomes originating from "Chinese spring" of the same group as the chromosome carrying the *Stb* gene in bread wheat (**Table 3-2**). A total of 69 crosses were made in glasshouse conditions, generating a total of 631 F_1 hybrid seeds (**Table 3-9**). The C15 population [hexaploid genotype "Synthetic 6X" x with LD-14 line: 7D (7B)] had the highest average F_1 seed produced per cross whereas the C14 population [bread wheat genotype "KK4500" x LD-1 line: 1D (1A)] had the lowest average F_1 seed produced per cross. No big differences were observed between crosses of the same hexaploid parent to either of the two LND-DS lines carrying the same pair of D-genomes substituting either the A or the B-genomes (**Table 3-9**). However, greater differences were observed in the average F_1 seed set per cross between the crosses using different hexaploid parents. Hence, the choice of the hexaploid wheat parent seems to play a role in the successful production of F_1 pentaploid progeny in the pentaploid crosses using LND-DS lines.

Table 3-9. The production of the F_1 hybrid seed of the crosses between bread wheat and LND-DS lines.

Pop. Ref.	F_1 pedigree	No. of crosses	No. of F_1 seed produced	Average seed set/cross
C14	KK4500/LD-1(1D/1A)	11	60	5
C18	KK4500/LD-10(1D/1B)	12	73	6
C10	SH M3/LD-4(3D/3A)	18	144	8
C9	SH M3/LD-11(3D/3B)	8	88	11
C11	Synthetic 6X/LD-7(7D/7A)	11	136	12
C15	Synthetic 6X/LD-14(7D/7B)	9	130	14
Total		**69**	**631**	**-**

Eight randomly selected F_1 pentaploid seed of each combination were germinated. However, only 2 to 6 seeds / combination successfully germinated and reached the adult stage (**Table 3-10**). This low germination rate was expected due to the unstable genomic composition of the F_1 plants (35 chromosomes characterised by the presence of 8 univalent D-genome chromosomes and the absence of an A- or B- chromosome depending on the LND-DS line used). Out of 106 crosses made, a total of 742 F_1T seeds were produced (**Table 3-10**). Ten randomly selected F_1T seeds of each combination were germinated and plants were back-crossed to the recurrent durum wheat parent to increase the durum wheat genomic background in the progeny. A total number of 1056 BC_1-F_1 seeds were produced out of 157 crosses made.

Table 3-10. F_1T and the BC_1-F_1 seed production of the 3 way crosses in glasshouse conditions.

Pop. Ref.	F_1T Pedigree	F_1T			BC_1-F_1		
		No. of F_1 plants crossed	No. of crosses	No. of F_1T seed produced	No. of F_1T plant back-crossed	No. of crosses	No. of BC_1-F_1 seed produced
C9-K	SH M3/LD-11/2/Karim	3	8	44	10	16	128
C9-O	SH M3/LD-11/2/Om Rabiaa 5		7	30	7	10	83
C10-K	SH M3/LD-4/2/Karim	2	3	15	7	6	34
C10-O	SH M3/LD-4/2/Om Rabiaa 5		2	11	2	4	20
C11-K	Synthetic 6X/LD-7/2/Karim	4	16	164	10	24	146
C11-O	Synthetic 6X/LD-7/2/Om Rabiaa 5		12	132	9	14	92
C15-K	Synthetic 6X/LD-14/2/Karim	6	13	69	7	12	69
C15-O	Synthetic 6X/LD-14/2/Om Rabiaa 5		15	43	10	14	110
C14-K	KK4500/LD-1/2/Karim	5	11	82	10	17	127
C14-O	KK4500/LD-1/2/Om Rabiaa 5		10	91	10	14	98
C18-K	KK4500/LD-10/2/Karim	3	4	34	8	8	45
C18-O	KK4500/LD-10/2/Om Rabiaa 5		7	37	10	18	104
Total		**23**	**106**	**742**	**101**	**157**	**1056**

Eight randomly selected BC_1-F_1 seeds from each of the 12 cross-combinations were germinated. A total of 49 plants out of the 11 populations reached maturity and set BC_1-F_2 seed (the number of BC_1-F_1 plants varied between three to seven per population) (**Table 3-11**). Most of the seed from the C10-O (SH M3/LD-4/2/Om Rabiaa 5) population failed to germinate while plants from the germinated ones had hybrid necrosis and died. A total of 4169 seeds were produced with a variable average seed set/plant between the different populations. The C14-O population had the highest seed set rate followed by the C15-K population. On the other hand, the C10-K population had the lowest BC_1-F_2 seed set (**Table 3-11**).

Table 3-11. BC_1-F_2 seed production of the three way crosses in glasshouse conditions.

Cross ref.	Pedigree	No. of plant self-pollinated	BC_1-F_2 seed produced	Average seed-set/plant
C9-K	SH M3/LD-11/2/Karim	6	602	100
C9-O	SH M3/LD-11/2/Om Rabiaa 5	3	370	123
C10-K	SH M3/LD-4/2/Karim	4	137	34
C11-K	Synthetic 6X/LD-7/2/Karim	6	309	52
C11-O	Synthetic 6X/LD-7/2/Om Rabiaa 5	4	156	39
C14-K	KK4500/LD-1/2/Karim	5	405	81
C14-O	KK4500/LD-1/2/Om Rabiaa 5	6	819	136
C15-K	Synthetic 6X/LD-14/2/Karim	3	378	126
C15-O	Synthetic 6X/LD-14/2/Om Rabiaa 5	2	217	109
C18-K	KK4500/LD-10/2/Karim	3	269	90
C18-O	KK4500/LD-10/2/Om Rabiaa 5	6	557	93
Total		**49**	**4169**	**-**

Depending on the number of spikes per plant, one to three heads of every F_1T plants were allowed to self-pollinate and produced a total of 1919 F_2T seeds (**Table 3-12**). The seed set per spike varied between the different populations. The C9-K population had the highest seed set/spike, indicating a high level of fertility. The C10-K and C10-O populations both involving the synthetic hexaploid wheat parent SH M3 with LD-4 and either of the two durum parents "Karim" and Om Rabiaa 5", respectively, had the lowest seed set (**Table 3-12**). This low seed set was also observed in the F_1T and the BC_1-F_1 generations for these two cross-combinations (**Table 3-10**, **Table 3-11**). In comparison to the rest of the crosses, this result indicate a low compatibility between the pentaploid F_1-C10 (SH M3/LD-4) produced and either of the durum wheat parents.

Table 3-12. F_2T seed production of the three-way crosses in glasshouse conditions.

Pop. Ref.	Pedigree	No. of heads self-pollinated	No. of F_2T seeds produced	Average seed-set/spike
C9-K	SH M3/LD-11/2/Karim	17	803	47
C9-O	SH M3/LD-11/2/Om Rabiaa 5	8	226	28
C10-K	SH M3/LD-4/2/Karim	2	19	10
C10-O	SH M3/LD-4/2/Om Rabiaa 5	1	1	1
C11-K	Synthetic 6X/LD-7/2/Karim	11	178	16
C11-O	Synthetic 6X/LD-7/2/Om Rabiaa 5	6	104	17
C14-K	KK4500/LD-1/2/Karim	11	139	13
C14-O	KK4500/LD-1/2/Om Rabiaa 5	6	133	22
C15-K	Synthetic 6X/LD-14/2/Karim	9	163	18
C15-O	Synthetic 6X/LD-14/2/Om Rabiaa 5	4	53	13
C18-K	KK4500/LD-10/2/Karim	2	29	15
C18-O	KK4500/LD-10/2/Om Rabiaa 5	6	71	12
Total		**83**	**1919**	**-**

3.3.2 Cytogenetic analysis of lines out of the two crossing strategies

3.3.2.1 Genomic composition of the screened lines

The genomic composition of 150 randomly selected lines from the BC_1-F_2 and BC_2-F_1 generations for the pentaploid bread wheat/durum wheat populations (first crossing strategy) and the F_2T and BC_1-F_1 generations for the 3 way-crosses involving the LND-DS lines (second crossing strategy) were screened using a cytological approach. Overall, only 82% (123 out of the 150 lines) of the lines were successfully screened with mc-GISH. No good quality metaphase spread or alternatively a clear mc-GISH photo could be established for the other 18% of the lines. Results of the successfully screened lines indicated that the majority of the lines had lost all D-genome chromosomes in both crossing strategies at all generations (**Figure 3-4**). The rest of the lines contained a varied amount of the D-genome as either full chromosome(s), telomeres or translocations (**Table 3-13**). A higher percentage of lines that had lost the D-genome was observed in both of the generations studied from the first

crossing strategy (**Figure 3-4**). A higher D-genome retention rate was obtained in lines of the three way crosses.

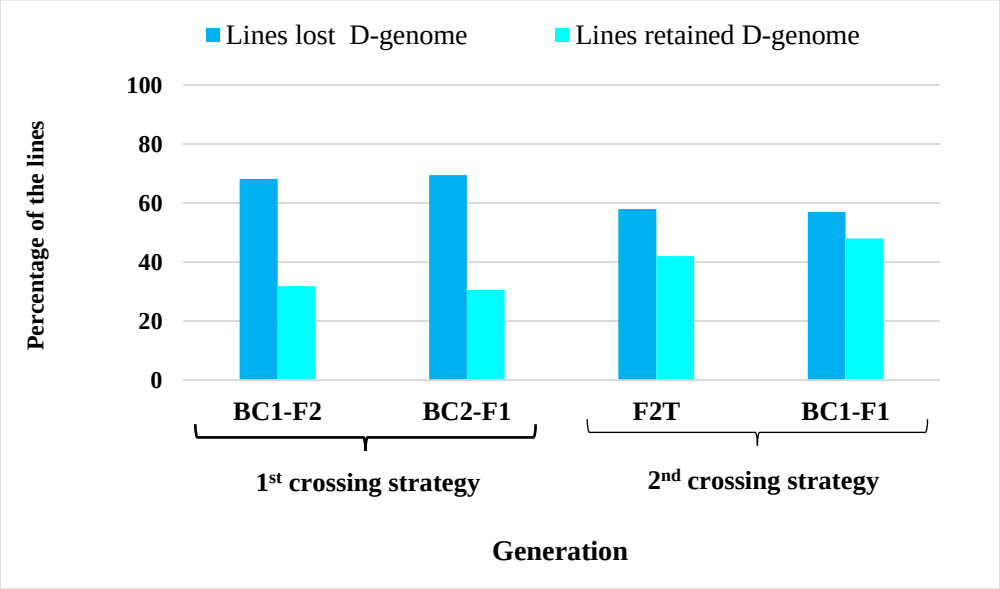

Figure 3-4. D-genome retention in the BC_1-F_2 and BC_2-F_1 generations and the F_2T and the BC_1-F_1 generations of the first and the second crossing strategy, respectively.

All lines from the first crossing strategy contained a complete set of A- and B-genome chromosomes. Some of the lines also retained between one to three D-genome chromosomes in the form of additions with a total chromosome number varying between 29 to 31 chromosomes.

Lines produced out of the second crossing strategy had retained between one to five D-genome chromosomes with a total chromosome number varying from 28 to 32 chromosomes (**Table 3-13**). Some of these lines also had a univalent A- or B-genome chromosomes missing, expected to be from the same group as the substituted A- or B-genome chromosomes in the parental LND-DS line used to generate the F_1. In addition, seven tetraploid lines with a monosomic D-genome substitution were identified in the BC_1-F_1 generation of the C10-K (1 line), C11-K (1 line) and C14-K (2 lines) populations and the F_2T generation of the C11-K (1 line), C14-K (1 line) and C18-O (1 line) populations. In all of these tetraploid lines the retained D-genome chromosome is expected to be substituting the corresponding A- or B-genome chromosomes substituted initially in the LND-DS line used (e.g. **Figure 3-5**).

Table 3-13. Genomic composition and intergenomic translocation revealed by mc-GISH analysis.

	Gene.	Cross ref.	No. of plants screened	No. of D-chrom. retained	Total chrom. No.	A or B chrom. missing*[No.]
First crossing strategy	BC_1-F_2	**C1**	4	0-2	28-30	0
		C3	8	0-1	28-29	0
		C4	8	0	28	0
		C7	7	0-1	28-29	0
		C8	5	0-1	28-29	0
	BC_2-F_1	**C1**	4	0-1	28-29	0
		C3	2	0	28	0
		C4	5	0	28	0
		C7	8	0-3	28-31	0
		C8	2	0-1	28-29	0
Second crossing strategy	BC_1-F_1	**C9-K**	5	0	28	0
		C9-O	3	0	28	0
		C10-K	4	0-3	28-31	3A*[1]
		C11-K	4	0-2	28-29	7A*[1]
		C11-O	3	4-5	30-32	7A*[1]
		C14-K	4	0-1	28-29	1A*[1]
		C14-O	2	0	28	0
		C15-K	2	0	28	0
		C15-O	3	1-3	29-31	0
		C18-K	2	0	28	0
		C18-O	4	0-2	28-30	1B*[1]
	F_2T	**C9-K**	6	0-1	29-29	0
		C9-O	4	1-3	29-31	3B*[1]
		C10-O	1	0	28	0
		C11-K	2	t-1*	28	7A*[1]
		C11-O	1	t	28	0
		C14-K	2	0-2	28-29	1A*[1]
		C14-O	4	0	28	0
		C15-K	5	0-1	28-29	0
		C15-O	4	0-2	28-30	0
		C18-K	3	0-1	28-29	0
		C18-O	2	1	28	1B*[1]

NB: t=telomere, *number of chromosome copies

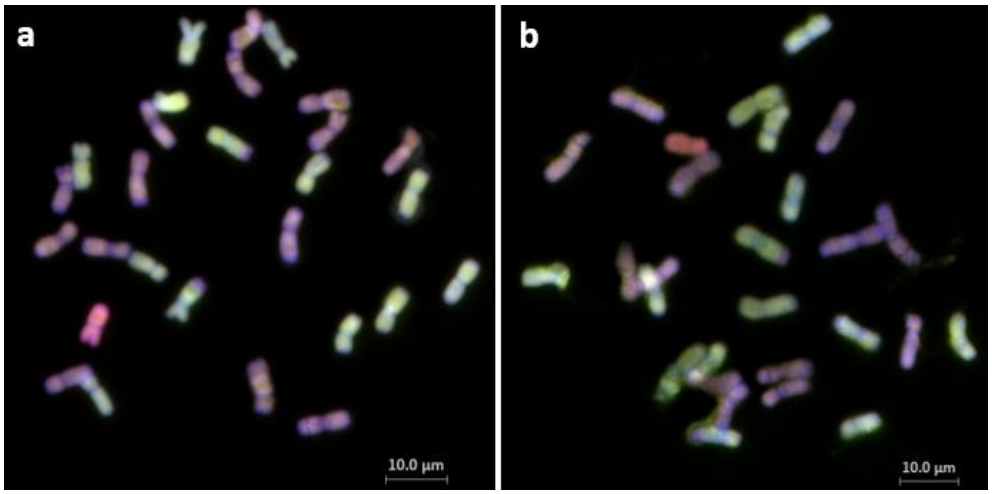

Figure 3-5. Mc-GISH of root-tip metaphase spreads of the tetraploid (a) BC_1-F_1-C11-K and (b) F_2T-C14-K lines showing a monosomic D (A) and D (B) substitutions, respectively.

3.3.2.2 Intergenomic spontaneous translocations involving the D-genome

Mc-GISH analysis revealed the presence of spontaneous genomic translocations between the D- and the A-genomes in 3% (4 lines) of the successfully screened lines.

Table 3-14. Chromosomal constitution of the lines showing spontaneous intergenomic rearrangement involving the D-genome.

Crossing strategy	Population	A-Chrom. No.	B-Chrom. No.	D-Chrom. No.	Transl-ocation *	Total chrom. No.
1st	**BC_1-F_2-C1**	14	14	t	D-a	29
	BC_1-F_2-C3	13	14	0	A-d	28
2nd	**F_2T-C11-KR**	13	14	t	D-a	28
	F_2T-C14-OR	14	14	0	A.D	29

NB: t=telomere,* '-' for paracentric translocation, '.' For centromeric translocation.

Two translocations were identified in the BC_1-F_2 generation of the C1 and C3 populations from the direct bread/durum wheat crosses (**Figure 3-6**). The first translocation identified in one of the BC_1-F_2-C1 lines was characterised as a small A-genome segment translocated in the long arm of a D-genome chromosome designed as "D-a" (NB: a lower case letter is used for the small segment and an uppercase letter for the larger segment). This translocation was present as an addition, alongside a D-telomere in a total of 29 chromosomes (**Figure 3-6a**). The second translocation was identified in one of the BC_1-F_2-C3

lines and characterised as the translocation of a small D-genome segment into the distal end of the short arm of an A-genome chromosome, designated as "A-d", (**Figure 3-6b**) in a total of 28 chromosomes.

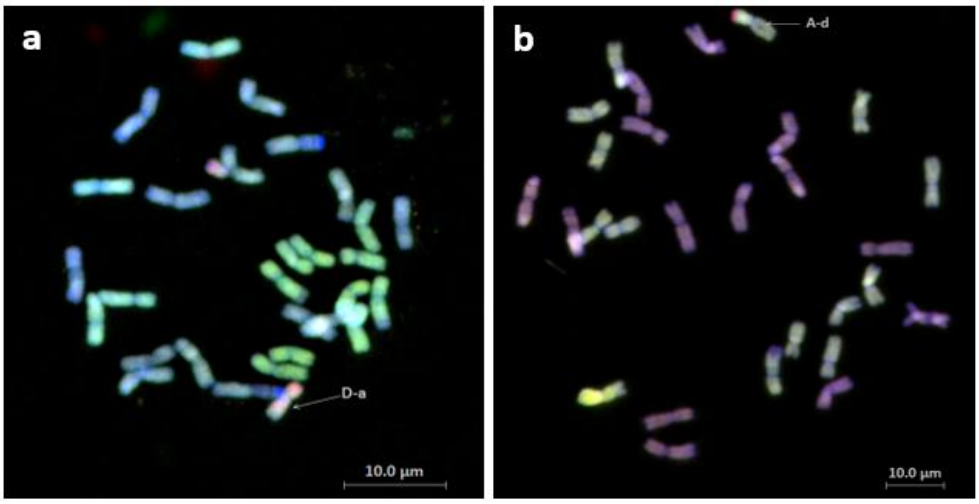

Figure 3-6. Mc-GISH of root-tip metaphase spreads of the (a) BC_1-F_2-C1 and (b) BC_1-F_2-C3 lines showing the presence of a "D-a" and an "A-d" genomic translocations, respectively.

Two more translocations, characterised as a telomeric "D-a" and a centromeric "A.D" translocation, were identified in two F_2T lines in the C11-K and C14-O populations, respectively (**Table 3-14**, **Figure 3-7**). The "D-a" translocation was introgressed into 28 chromosomes alongside a D-genome telomere. The centromeric A.D translocation was present as an addition, in a total of 28 chromosomes (**Figure 3-7b**).

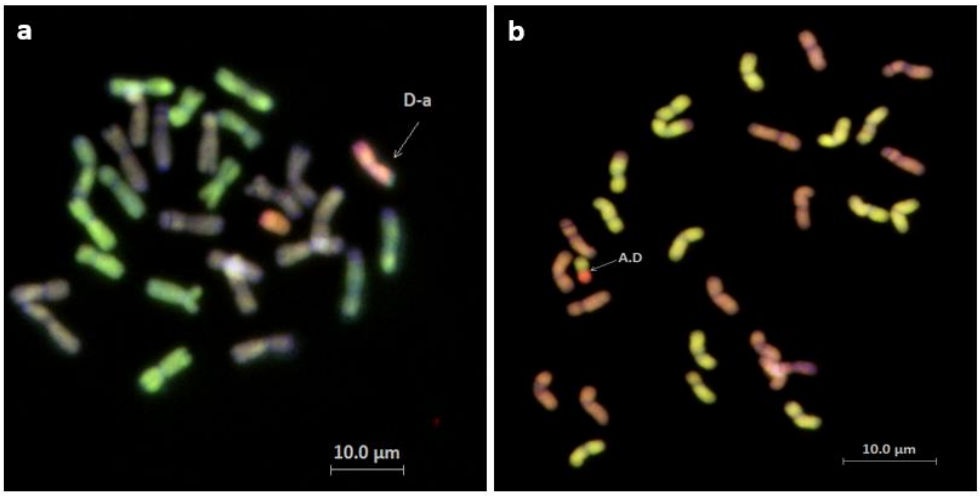

Figure 3-7. Mc-GISH picture of root-tip metaphase spreads of the (a) F_2T-C11-K and (b) F_2T-C14-O lines showing the presence of a "D-a" and an "A.D" genomic translocation, respectively.

3.3.3 Screening for STB disease resistance under field conditions

3.3.3.1 First crossing strategy: Phenotyping of the F_1 and the BC_1-F_1 generations

The F_1 plants of each combination showed a complete resistance with a total absence of disease symptoms. This indicates that the resistance might be dominant and controlled by more than one gene. Segregation for STB disease started in the BC_1-F_1 generation after backcrossing to the recurrent durum wheat parent. From 394 BC_1-F_1 plants, 63 showed complete STB resistance (**Table 3-15**). The rest of the plants showed a high level of resistance - equivalent or better than the local resistant check "Salim" that shows a 45% SDS. (**Table 3-15**).

Table 3-15. Plant selection of the interspecific hybrid populations for STB resistance in field conditions.

Pop. Ref.	Pedigree	Total No. of plants	No. of R plants	No. MR plants
BC_1-F_1-C1	KK4500/Karim*2	71	10	61
BC_1-F_1-C2	KK4500/Om Rabiaa 5*2	36	15	21
BC_1-F_1-C3	SH M3/Karim*2	25	6	19
BC_1-F_1-C4	SH M3/Om Rabiaa 5*2	47	2	45
BC_1-F_1-C7	Synthetic 6X/Karim*2	121	10	111
BC_1-F_1-C8	Synthetic 6X/Om Rabiaa 5*2	94	20	74
Total		**394**	**63**	**331**

NB: R=resistant (SDS=0%), MR=moderately resistant (SDS<30%).

3.3.3.2 Second crossing strategy: Phenotyping of the F_1 and the F_1T generations

All the plants screened from the six F_1 populations showed complete resistance with an absence of any disease symptoms. This complete resistance of the F_1 plants suggests that the resistance of the bread wheat to the Tunisian population of *Z. tritici* strains is probably dominant. This result is consistent with the resistance observed in the F_1 pentaploid plants from the cross of the same bread wheat genotypes with the two susceptible durum wheat parents.

All the F_1T populations (with the exception of the C10-K and C10-O populations) were phenotyped for STB disease under field conditions. The C10-

K and C10-O populations were excluded because of the limited amount of seed produced and their very low germination rate seen in the field. The rest of the ten F_1T populations segregated for STB disease resistance. Plants with a complete resistance to STB, as well as plants with a good level of resistance, were distinguished in these populations (**Table 3-16**). Out of 358 screened plants, 81 were completely resistant. A further 17 plants with a good resistance level, in which the disease scoring was lower than the local resistant check "Salim", were also selected (**Table 3-16**). This result, together with the segregation of the BC_1-F_1 plants (first crossing strategy) for STB disease, suggests that the resistance from the bread wheat is quantitative, i.e. controlled by more than one gene with additive effect.

Table 3-16. Plant selection of the F_1T populations of the second approach.

Pop. Ref.	Pedigree	Total No. of plants	No. of R plants	No. of MR plants
F_1T-C9-K	SH M3/LD-11/2/Karim	38	3	35
F_1T-C9-O	SH M3/LD-11/2/Om Rabiaa 5	16	4	12
F_1T-C11-K	Synthetic 6X/LD-7/2/Karim	61	8	53
F_1T-C11-O	Synthetic 6X/LD-7/2/Om Rabiaa 5	49	7	42
F_1T-C14-K	KK4500/LD-1/2/Karim	42	9	33
F_1T-C14-O	KK4500/LD-1/2/Om Rabiaa 5	36	12	24
F_1T-C15-K	Synthetic 6X/LD-14/2/Karim	32	12	20
F_1T-C15-O	Synthetic 6X/LD-14/2/Om Rabiaa 5	45	12	33
F_1T-C18-K	KK4500/LD-10/2/Karim	14	5	9
F_1T-C18-O	KK4500/LD-10/2/Om Rabiaa 5	17	9	8
Total		**358**	**81**	**269**

NB: R=resistant, MR=moderately resistant.

3.3.4 *Stb* gene transfer and their effect on STB disease resistance in durum wheat

3.3.5 Plant phenotyping

A total of 665 plant out of five advanced backcrossing populations, in the BC_1-F_3 (populations: C3 and C4) and the BC_1-F_2 (populations: C7, C9-K and C18-K) stage were chosen to study for the potential transfer of the *Stb* genes

and their effect on disease resistance in durum wheat. Plants were screened individually for STB resistance using the double-digit scale. Disease scores were transformed into percentage of Septoria disease severity (SDS) (Sharma and Duveiller, 2007). In this study plants with an SDS lower or equal to 30% were considered resistant (R), whereas plants with a SDS between 30 and 50% were considered moderately resistant (MR). Plants with an SDS above 50% were considered susceptible (S). Plants with improved STB resistance compared to the resistance check "Salim" (SDS=45%) were observed in all of the screened populations. The frequency of SDS distribution differed between the six different populations (**Figure 3-8**). A higher percentage of R plants (<30% SDS) compared to S plants was observed in all the populations screened from both crossing strategies (**Table 3-17**). These results suggest that the crosses are promising and have the ability to improve STB disease resistance in durum wheat.

Table 3-17. Percentage of the R, MR and S plants for STB disease resistance in six populations tested field conditions.

Crossing strategy	Generation	Pop. ref.	% of R plants	% of MR plants	% of S plants
1st crossing strategy	BC_1F_2	**C1**	66	17	17
		C7	58	28	14
	BC_1F_3	**C3**	54	43	3
		C4	45	32	23
2nd crossing strategy	BC_1F_2	**C9-K**	33	32	35
		C18-K	56	32	12

NB: R=resistant, MR=moderately resistant, S=susceptible.

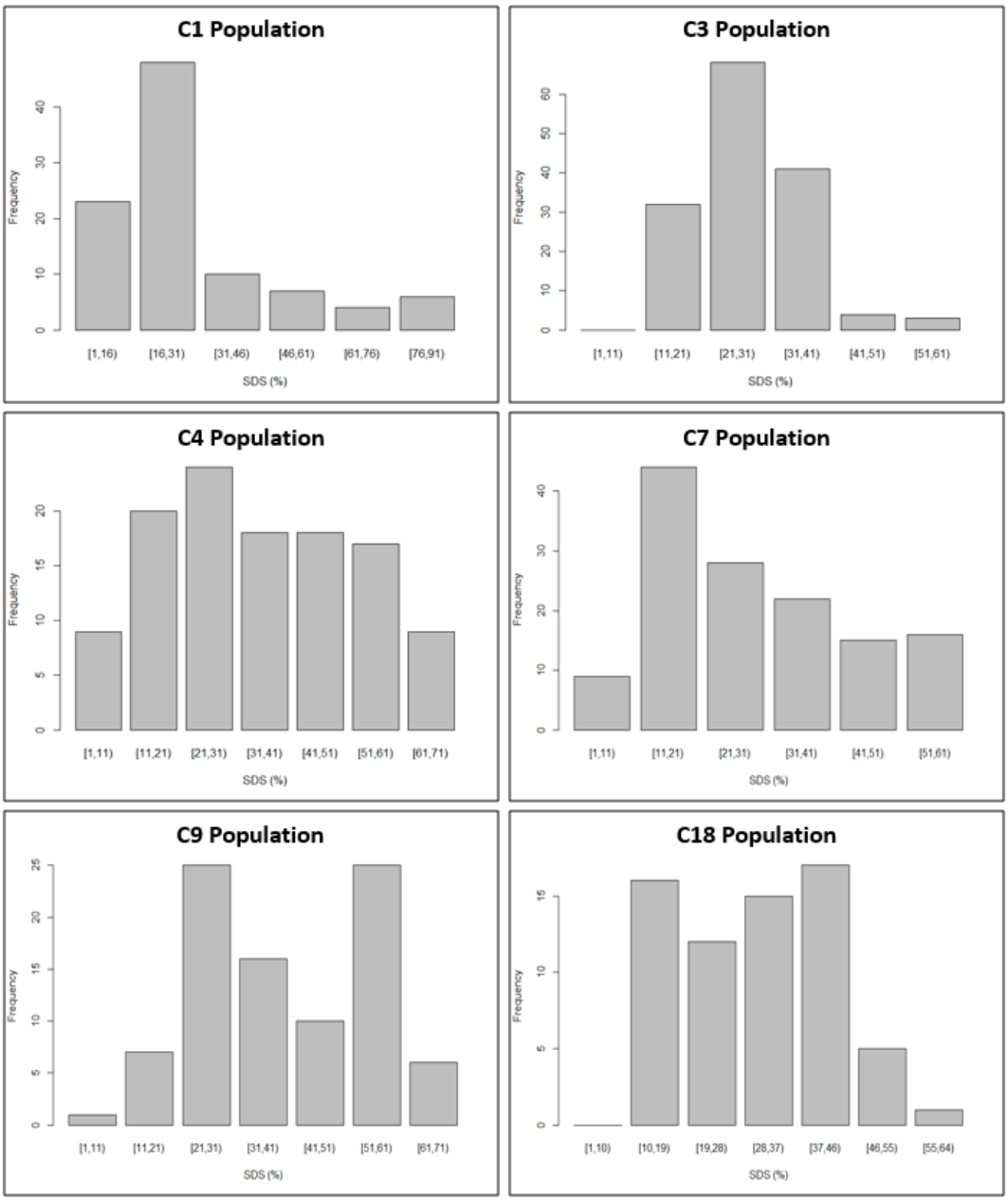

Figure 3-8. Frequency distributions of the Septoria disease severity in the C1 and C7 populations in the BC_1-F_2 generation, the C3 and C4 populations in the BC_1-F_3 generation and the C9 and the C18 populations in the BC_1-F_2 generation.

3.3.6 The use of LND-DS lines to assess the effect of the D-genome on STB disease in seedling and adult stage

With the aim of assessing the effect of the disomic D-genome substitution on STB disease resistance in durum wheat, the full set of the LND-DS lines (14 lines) alongside the "Langdon" genotype were phenotyped for STB disease reaction at the seedling and adult stages under controlled and field conditions, respectively (**Figure 3-9**). In comparison to the "Langdon" genotype that showed a good level of STB resistance at both stages (SDS<30%), a high level

of variation for STB disease response of the LND-DS lines was recorded at both stages (**Figure 3-9**).

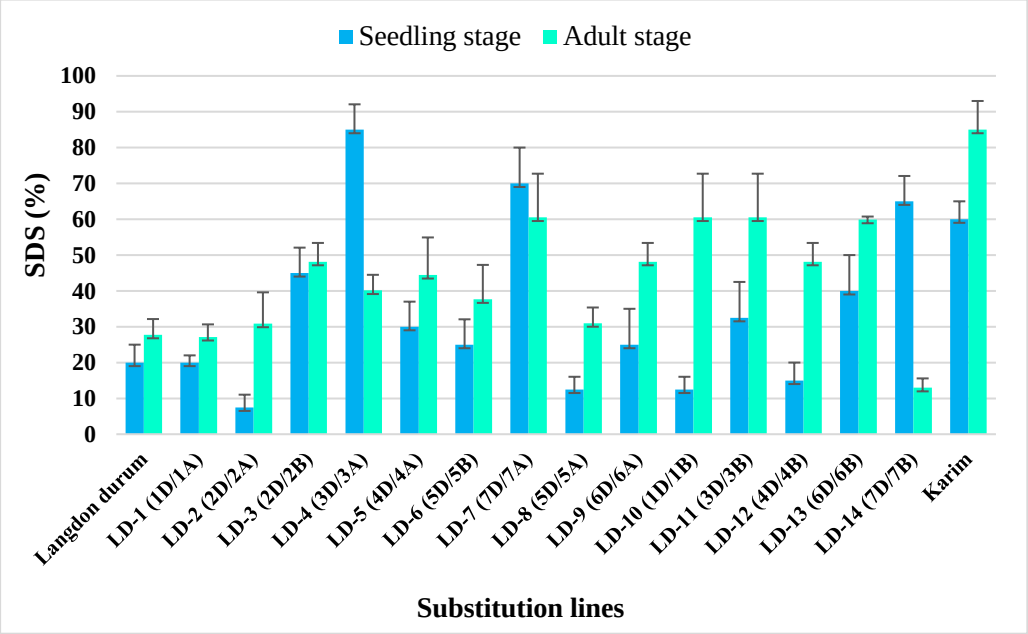

Figure 3-9. Langdon D-genome substitution lines reaction to STB at seedling and adult stage tested under controlled and field conditions, respectively.

3.3.6.1 Reaction of LND-DS lines to STB at the seedling stage

At the seedling stage, LD-4, LD-7 and LD-14 substitution lines showed the highest disease severity, that was found to be higher than the susceptible check "Karim" (SDS>60%). Thus, the 7D substitution of either the 7A or 7B pair of chromosomes in the LD-7 and LD-14 lines, respectively, increased disease susceptibility (**Figure 3-9**). In this case we can conclude that the 7D chromosome my carry a susceptibility gene or a resistance suppressor gene expressed at the seedling stage. The high susceptibility of line LD-4 carrying the 3D (3A) substitution might be associated with the absence of the pair of 3A chromosomes compared to line LD-11 carrying the 3D (3B) substitution that showed a STB reaction more or less similar to the "Langdon" genotype. Hence, chromosome 3A may harbour a resistance gene. Substitution lines LD-2, LD-8 and LD-10 had a lower disease severity than "Langdon" (SDS<15%). The substitution lines carrying the same pair of D-genome chromosomes, however, showed a higher disease severity and thus these results show that the resistance in these lines is probably related to the A- and the B-genomes rather than the D-genome. For example, the resistance reaction of line LD-2 and the susceptible

reaction of line LD-3, carrying the same pair of 2D chromosomes substituting a pair of 2A and 2B chromosomes, respectively, indicate the presence of a partial resistance gene on chromosome 2B due to the increase of the SDS mean score by 25% compared to "Langdon" when it is absent. The absence of chromosome 2A was accompanied by a decrease of 8% in disease severity suggesting that this chromosome may harbour either a susceptibility or a resistance suppressor gene. The rest of the lines had a disease severity similar to the check, e.g. lines LD-1 and LD-12 (20% SDS) or slightly higher, e.g. LD-3, LD-5, LD-6, LD-9 and LD-13 (25% < SDS < 45%) (**Figure 3-9**) showing that no major effect on disease resistance at the seedling stage can be attributed to the substituted chromosomes in these lines.

3.3.6.2 Reaction of LND-DS lines to STB at the adult stage

Screening of the LND-DS lines to STB disease at the adult stage was assessed during two consecutive growing seasons (2016/2017 and 2017/2018) under field conditions. With the exception of line LD-14, carrying the 7D (7B) substitution that showed the lowest disease severity (13% SDS) with a better resistance level than "Langdon" (28% SDS), none of the substitution lines had a better disease resistance level than "Langdon". All the lines had a lower SDS mean score than the susceptible check. In comparison with the LD-7 line carrying the 7D (7B) substitution, the improved level of resistance in the LD-14 line appears to be associated with the absence of the 7B chromosomes than to the presence of the 7D chromosome (**Figure 3-9**). Lines such as LD-7, LD-10, LD-11, and LD-13 had a high disease severity reaction (60% SDS) compared to the "Langdon" genotype, indicating that the substitutions in these lines had a negative effect on STB disease resistance.

As with the observations at the seedling stage, none of the D-substitutions appeared to be related to the improvement in the level of disease resistance in the adult stage. The presence/absence of the A- and B-genome chromosomes seemed to have more effect on STB disease reaction in durum wheat. For instance, LD-10 line carrying the 1D (1B) substitution had a higher disease severity (60% SDS) compared to the LD-1 line carrying the 1D (1A) substitution that had a disease reaction similar to the resistant "Langdon" (**Figure 3-9**). The absence of the 1B chromosome appeared to increase the level

of disease severity at the adult stage, indicating the potential presence of a partial resistance gene on chromosome 1B.

3.3.6.3 Correlation between seedling and adult reaction of LND-DS lines to STB disease.

Pearson correlation analysis confirmed the complete absence of a correlation between the STB disease reaction of the LND-DS lines at the seedling and the adult stage (p-value: 0.963, Coefficient of correlation: 0.013). For instance, the LD-10 line carrying the 1D (1B) substitution which showed a low disease severity at the seedling stage (13% SDS), showed a higher susceptibility reaction (60% SDS) at the adult stage. The opposite was observed for line LD-14, carrying the 7D (7B) substitution, which was highly susceptible at the seedling stage but showed a high level of resistance in the adult stage (**Figure 3-9**). Only line LD-1, with the 1D (1A) substitution, had a good level of resistance at both the seedling and adult stages (similar to the resistant "Langdon" check) thus showing no major effect caused by this particular substitution on STB disease resistance.

3.3.7 Molecular analysis for the retention of the *Stb* genes

Using SSR markers closely linked to the *Stb* genes, 665 lines from the six different populations were screened alongside the parental lines (Supp. material 1 to 7). Data for the *Stb16* genes were excluded as the associated marker showed monomorphic behaviour. Unfortunately, none of the *Stb* gene located on the D-genome (S*tb10* and *Stb5*) could be identified in the screened progenies. *Stb6* and *Stb17* genes were identified in the progeny of more than one population. A very high retention rate of *Stb17* (97% and 95% in lines BC_1-F_3-C3 and BC_1-F_2-C9-K, respectively) was observed compared to the rest of the *Stb* genes (**Table 3-18**).

Using the first crossing strategy, *Stb6*, originally present in "KK4500" and "Synthetic 6X", was transferred to the BC_1-F_2 progeny of the C1 and the C7 populations, in a tetraploid background of both durum wheats used. In addition, *Stb17,* originally present in the SH M3 bread wheat genotype, was identified in 97% and 27% of the BC_1-F_3 progeny screened from the C3 and C4 populations, out of the crosses of "SH M3" with "Karim" and "Om Rabiaa 5", respectively. *Stb7* and *Stb12*, originally present in the bread wheat genotype "KK4500", were

found to be present in both Karim and Om Rabiaa 5 and thus were present in all the screened progeny from the crosses between "KK4500" and either of the durum wheat genotypes (**Table 3-18**). *Stb7* and *Stb12* are therefore not effective against *Z. tritici*-durum wheat specific strains and have no effect on STB disease resistance in durum wheat.

The screening results of the C9-K and C18-K populations of the three way crosses showed they had failed to retain the *Stb* genes located on the D-genome. In the progeny selected, the use of LND-DS lines in the crossing strategy did not help to pull the D-genome chromosome carrying the *Stb* gene into the BC_1-F_2 generation after twice crossing to the durum wheat parent. Only the *Stb17* gene located on the A-genome was retained in 95% of the BC_1-F_2-C9-K population (**Table 3-18**).

Table 3-18. The percentage of *Stb* genes retention in the progeny of six populations involving bread wheat with durum wheat.

Crossing strategy	Pop. Ref.		*Stb* gene	Locus	% of plants retained the *Stb* gene	% of plants lost the *Stb* gene
First crossing strategy	BC_1-F_2	C1	***Stb6***	3AS	15	85
			Stb7/stb12	4AL	100	0
			Stb10	1Dc	0	100
		C7	***Stb6***	3AS	17	83
			Stb5	7DS	0	100
	BC_1-F_3	C3	***Stb17***	5AL	97	3
			Stb16	3DL	--	--
		C4	***Stb17***	5AL	24	76
			Stb16	3DL	--	--
Second crossing strategy	BC_1-F_2	C9-K	***Stb17***	5AL	95	5
			Stb16	3DL	--	--
		C18-K	***Stb7/stb12***	4AL	100	0
			Stb10	1Dc	0	100

--monomorphic marker

3.3.8 Effect of *Stb* genes transfer on STB disease resistance in durum wheat

3.3.8.1 Correlation analysis between Septoria disease severity and plant height

An association between plant height and susceptibility (Rosielle and Brown, 1979; Danon *et al.*, 1982), and between heading time and susceptibility (Eyal, 1981; Rosielle and Boyd, 1985) has been observed in the STB-wheat pathosystem. Many scientists reported increased disease severities in earlier heading and shorter cultivars (Eyal *et al.*, 1983; Eyal *et al.*, 1987; Van Beuningen and Kohli, 1990; Jlibene *et al.*, 1992; Camacho-Casas *et al.*, 1995; Arama *et al.*, 1994; 1999). Therefore, to avoid the confounding effects of height and flowering time on disease severity, during this study, depending on the number of plants available per population, only early heading, short plants with a durum wheat head type were selected. Nevertheless, plant height measurements for all individual plants were recorded. Pearson correlation analysis between plant height and SDS in all of the studied populations showed a significant negative correlation in only two of the populations; the C1 (P-value=0.01) and C4 (P-value=0.002) (**Table 3-19**). However, the R squared values appeared very low for these two populations (r^2=0.2 for the C1 and r^2=0.08 for the C4) which suggest a weak association between the two traits. Thus height only explains a very small amount of the variation in resistance. No significant correlation between plant height and disease severity was distinguished for the rest of the populations (**Table 3-19**, **Figure 3-10**).

Table 3-19. Pearson correlation and R-squared analysis between Septoria disease severity and plant height.

Population reference	Generation	Pop. size	df	R^2	P-value	Corr. Coef.
C1	BC_1-F_2	99	27	0.2005	0.0148*	-0.4477
C3	BC_1-F_3	150	142	0.0006	0.7654	0.0250
C4	BC_1-F_3	119	109	0.0832	0.0021*	-0.2886
C7	BC_2-F_2	138	135	0.0070	0.3281	-0.0841
C9	BC_1-F_2	91	89	0.0272	0.1177	-0.1651
C18	BC_1-F_2	68	66	0.0091	0.439	-0.0953

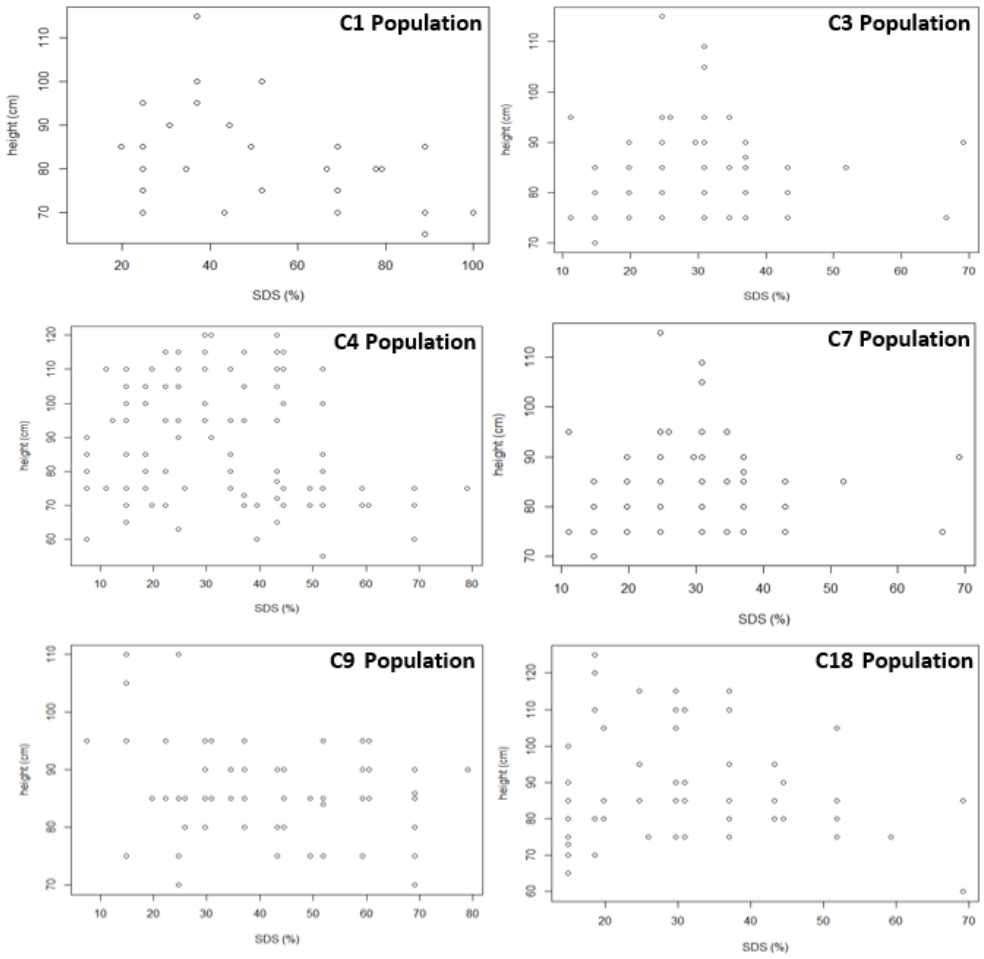

Figure 3-10. Scatter plot of Septoria disease severity (SDS) versus plant height in the six populations.

3.3.8.2 Correlation analysis between Septoria disease severity and the transferred *Stb* genes

Chi-squared analysis between the presence/absence of *Stb* genes and STB disease severity were carried out on the five populations in which the *Stb6* and/or the *Stb17* were transferred. Results showed no correlation between the presence of *Stb* genes and disease resistance (**Table 3-20**) and thus the transfer of *Stb6* and *Stb17* into the two susceptible durum genotypes had no effect on disease resistance. Hence, these *Stb* resistance genes previously mapped in bread wheat using bread wheat specific isolates, cannot be used in durum wheat improvement.

Table 3-20. Chi-squared analysis for Septoria disease severity and presence/absence of *Stb* genes.

Population reference	*Stb* gene	X^2 value	df	P-value
BC_1-F_2-**C1**	*Stb6*	6	4	0.1991
BC_1-F_2-**C7**	*Stb6*	6	4	0.1991
BC_1-F_3-**C3**	*Stb17*	6	4	0.1991
BC_1-F_3-**C4**	*Stb17*	6	4	0.1991
BC_1-F_2-**C9-K**	*Stb17*	3	2	0.2231

3.3.9 Genomic inheritance in populations derived from hexaploid/ tetraploid crosses: first crossing strategy

The populations C1, C3, C4 and C7, already screened for the retention of *Stb* genes from the bread wheat parents, were genotyped using Diversity Arrays Technology (DArTseq™) markers. A total of 55,375 sequences were generated across the whole wheat genome, of which only 43,444 markers were allocated to a specific chromosome. The average call rate for each sequence was 85%. Flapjack software (V10.0.1) was used to select those sequences that were polymorphic between the two parents in every population. Sequences that were non-consistent within the replicates from the same parental lines and sequences that had missing values in more than 80% of the lines were removed. The final number of sequences used in the analyses varied between 2,292 and 6,789 depending on the population, with the lowest number of markers obtained for the D-genome (**Table 3-21**).

Table 3-21. Number of polymorphic SNP markers selected per genome in every population.

Population reference	BC_1-F2-C1	BC_1-F_3-C3	BC_1-F_3-C4	BC_1-F_2-C7
Population size	107	150	106	124
A-genome	2252	1173	837	2446
B-genome	2603	1093	1007	2779
D-genome	1427	532	448	1564
Total	**6282**	**2798**	**2292**	**6789**

3.3.10 Retention of the A- and B-genome bread wheat alleles

Using the marker assisted backcrossing analysis of the Flapjack software, that takes into consideration the presence/absence of the SNP marker call in the progeny compared to the parental lines, the genomic proportions of the A-, B- and D-genome alleles in every lines could be quantified. Results showed that the genomic A and B proportion inherited from the durum wheat parent was higher than that from the bread wheat parent. The overall mean proportion of A and B alleles inherited from the bread wheat parent didn't exceed 32% in any of the populations although the mean proportion did vary between the different populations (**Table 3-22**, **Figure 3-11**). In the BC_1-F_3 generation, the highest mean A- and B-genome bread wheat alleles retained was in the C3-population (32%) where the durum parent was "Om Rabiaa 5". In comparison, the C4 population that shares the same bread wheat parent with the C3 population but had "Karim" as the durum parent, had retained only 17% of the A- and B-genome bread wheat alleles. This result suggests that the choice of the durum genotype might play a role in the inheritance of the bread wheat genome in the advanced backcrossing generations of the pentaploid cross to the durum wheat. In the BC_1-F_2 generation, however, the C1 population had a slightly higher retention rate (18%) of the A- and B-genome bread wheat allele compared to the C7 population (17%). These two populations share the same durum wheat parent "Karim" crossed to the bread wheat genotypes "KK4500" and "Synthetic 6X" respectively. This would suggest that the choice of bread wheat parent has only a minor effect on the inheritance of the bread wheat genome.

Table 3-22. Average percentage of the A- and B-genome bread wheat allele's retention in the four studied populations.

Population reference	Average % of A- and B-genome retention
C1	18
C3	32
C4	17
C7	15

The pairwise *t* test indicated no significant differences between the mean proportion of the A and B alleles inherited from the bread wheat parent in the crosses (P =0.410) (**Figure 3-11**). Furthermore, single factor ANOVA analysis indicated a significant variation (P<0.001) in the proportion of bread wheat alleles inherited from individual chromosomes between the four populations (e.g. 1A, 2A, 3A, etc.) (**Figure 3-12**).

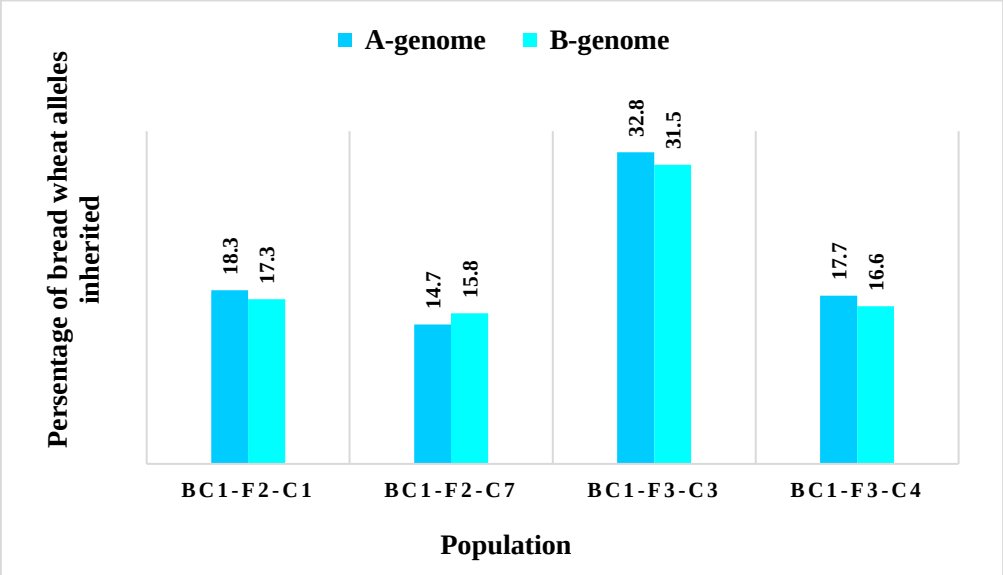

Figure 3-11. Average percentage retention of A- and B-genome bread wheat alleles in the four studied populations.

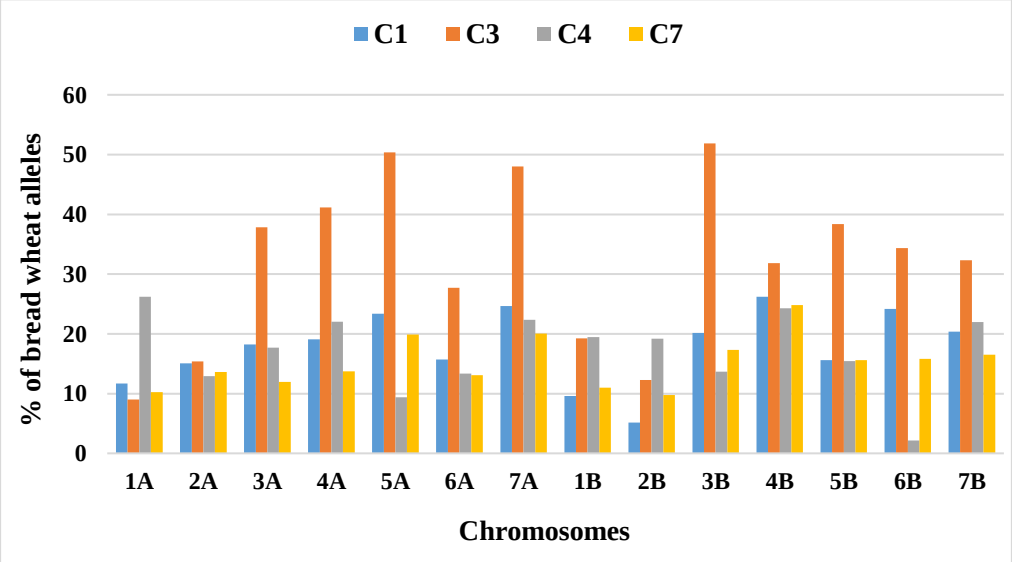

Figure 3-12. Percentage of A and B bread wheat alleles retained per individual chromosomes.

Individual lines had retained varying proportions of A- and B-genome alleles of the bread wheat parent ranging from 5.9% to 30.5% in the C1 population, 0.3

to 39 % in the C3 population, 4.2% to 56.6% in the C4 population and 8.3 to 41.2% in the C7 population (**Figure 3-13**). Only one line (with the highest mean proportion of A- and B-genome bread wheat alleles) was identified in the BC_1-F_3-C4 population. However, overall the BC_1-F_3 progeny of the C3 population had the highest retention rate of the A- and B-genome bread wheat alleles.

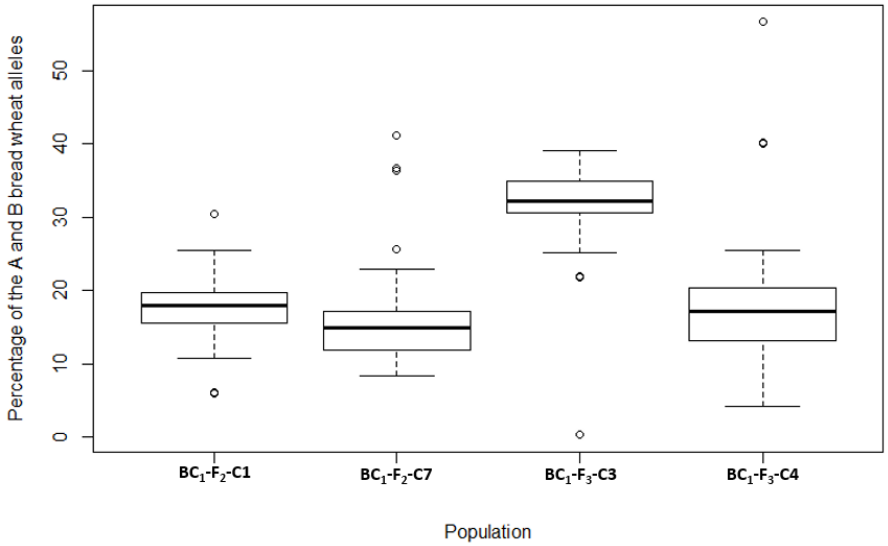

Figure 3-13. Boxplots representing the percentage of the A- and B-alleles retained from the bread wheat parent per population.

3.3.11 D-genome retention

A very low proportion of D-genome alleles were retained in all generations. The highest average retention was 4.2%, with an overall average of 2.9% per population (**Table 3-23**). This low proportion of D-genome retention was expected in the BC_1-F_2 and BC_1-F_3 generation after backcrossing to the durum parent. However, it does indicate that some lines retained D-genome introgressions, translocations, telomere(s) or full chromosomes.

Table 3-23. Average mean percentage of the D-genome allele's retention in the four studied populations.

Population reference	Percentage D-genome alleles retention
C1	2.5
C3	4.2
C4	2.4
C7	2.5
Average	**2.9**

Individual lines retained varying proportions of D-genome alleles ranging from zero to 13%, 49.5%, 10% and 15% in the C1, C7, C3 and C4 populations, respectively (**Figure 3-14**). Using Flapjack, the presence of a whole or at least a partial copy of the seven D-genome chromosome groups were identified in some of the lines (e.g. **Figure 3-15**). The presence of heterozygous calls for most of the alleles in these lines for a particular chromosome could indicate the presence of a single copy of the D-genome chromosome.

In total, 13 lines were seen to carry at least one D-genome chromosome (**Figure 3-16**). In the BC_1-F_2 generation, three lines belonging to the C1 population and two lines belonging to the C7 populations, retained the highest amount of the D-genome alleles at 49.5% and 38.6%, respectively and carried at least one copy of all seven D-genome chromosome groups (**Figure 3-16**). In the BC_1-F_3 generation, however, only one line from the C4 population had retained a copy of all seven D-genome chromosomes. The rest of the lines had each retained only one D-genome chromosome identified as either 3D, 4D or 5D, except for one line that retained six D-genome chromosomes (chromosome 1D was lost) (**Figure 3-16**).

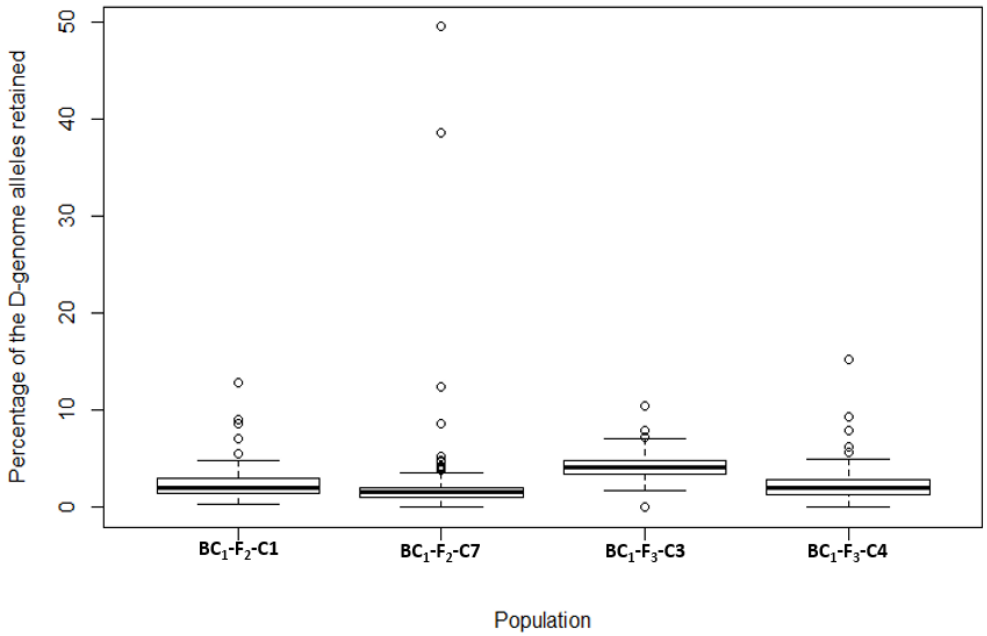

Figure 3-14. Boxplots representing the percentage of the D-genome alleles retained from the bread wheat parent per population.

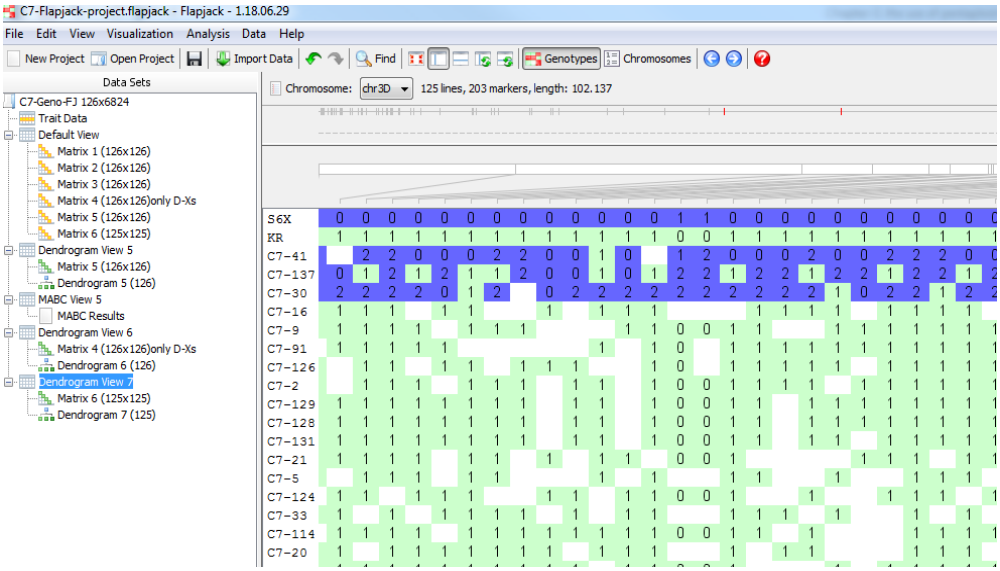

Figure 3-15. Flapjack interface shown the retention of 3D chromosome in the C7-41, C7-137 and C7-30 lines in the C7 population (S6X=bread wheat parent "Synthetic 6X", KR= durum wheat parent "Karim", blue: allele match to the S6X, light blue=allele match to KR, 0/1=SNP call, 2=heterozygous call).

Population	Line ref.	1D	2D	3D	4D	5D	6D	7D
BC_1-F2-C1	C1-7							
	C1-67							
	C1-71							
	C1-2							
	C1-3							
	C1-47							
BC_1-F_2-C7	C7-41							
	C7-137							
	C7-30							
BC_1-F_3-C3	C3-72							
BC_1-F_3-C4	C4-102							
	C4-109							
	C4-113							

Figure 3-16. Number of unique D-genome chromosomes retained in each line (Purple cells=presence, light blue cells=absence).

3.3.12 Correlation between D-genome retention and STB disease resistance

Some of the lines that lost all D-genome chromosomes still showed a good level of resistance to STB disease resistance, indicating that in these lines resistance appears to be more related to the A and/or the B genome from bread wheat. Pearson correlation analysis between the SDS and the percentage of the D-genome alleles retained showed the absence of significant correlation in the C1, C3 and C4 populations. A significant negative correlation (P-value= 0.05, Corr. Coef. = -0.177) was distinguished in only the C7 population (**Table 3-24**).

This result suggests the potential contribution of the D-genome alleles of the hexaploid genotype "Synthetic 6X" to STB resistance in durum wheat.

Table 3-24. Pearson correlation analysis between Septoria disease severity and the mean percentage of D-genome retention.

Population reference	df	P-value	Corr. Coef.
BC_1-F_2-C1	94	0.2259	-0.12473
BC_1-F_2-C7	121	0.05*	-0.1771
BC_1-F_3-C3	148	0.4422	0.0632
BC_1-F_3-C4	104	0.1011	-0.1601

*significant

Of the lines carrying the individual 3D, 4D and 5D chromosomes, the lowest SDS score was associated with the presence of chromosome 5D in the BC_1-F_2-C1 and BC_1-F_3-C4 populations (**Table 3-25**), suggesting that this particular chromosome may harbour an effective gene to *Z. tritici*-durum wheat specific strains contributing to the disease resistance. Relatively low disease infection was also associated with the presence of the 3D and 4D chromosomes. However, in all these lines, it is possible that the resistance may be contributed by the A and/or the B-genome of bread wheat. Therefore, fine mapping needs to be undertaken to confirm the resistance source.

Table 3-25. Septoria disease severity of lines shown the presence of individual D-genome chromosomes.

Population	Line ref.	D-genome chrom. retained	SDS (%)
BC_1-F_3-C4	C4-109	5D	11.11
BC_1-F_3-C4	C4-113	5D	12.35
BC_1-F_2-C1	C1-47	5D	14.81
BC_1-F_3-C7	C7-30	3D	18.52
BC_1-F_2-C1	C1-3	4D	19.75
BC_1-F_2-C1	C1-2	4D	30.86

3.3.13 Preliminary Quantitative Trait Loci (QTL) mapping for resistance to STB disease in the C1 population

In total 99 BC_1-F_2 lines were analysed for resistance to STB disease in field conditions and plant height. Lines were genotyped via GBS platform and the produced polymorphic SNP markers were selected and used to construct linkage maps with the MAP function in QTL IciMapping V4.1 based on the recombination frequency within the population. The map distance (centimorgans, cM) was converted by recombination fractions using the Kosambi function. The QTL locations and effects were analysed with QTL IciMapping V4.1 using inclusive composite interval mapping (ICIM) analysis method. The threshold LOD score was set at 2.5 (manual input) to detect significant QTLs. The phenotypic variances explained by individual QTLs were also obtained using ICIM (**Table 3-26**).

Four STB resistant minor QTLs were detected in the C1 population that explained from 5 to 6% of the phenotypic variation (**Figure 3-17**). Two of these QTLs on the 6A and 7A chromosome had negative value of additive effect of resistance allele indicating that the alleles were inherited from the susceptible durum wheat parent Karim. Another two minor QTL were located on the 4A chromosome and had a positive additive effect value indicating that these alleles were inherited from the resistant bread wheat parent KK4500. The Plant height QTL was located on the chromosome 1B were the known Rht-B1 gene is located indicating the absence of any pleiotropic effect between the two traits.

Table 3-26. Summary of STB resistance and plant height QTLs in the C1 population.

Trait Name	Chromosome	Left marker	Right marker	LOD	PVE*[a] (%)	Add*[b]
STB	4A	4329414|F|0--67:T>C	3959046|F|0--23:A>G	3.3419	5.1919	20.8201
STB	4A	1009996|F|0--33:C>T	4911139|F|0--11:G>A	2.9563	1.5843	7.7122
STB	6A	980758|F|0--60:A>G	4910157|F|0--22:C>T	4.412	6.392	-20.3722
STB	7A	1229978|F|0--20:T>C	4992599|F|0--41:C>T	4.0133	6.4764	-20.9879
Height	1B	100181549|F|0--24:T>C	100171886|F|0--32:G>C	2.5847	63.9151	-6.3089

*a Phenotypic variation explained by epsitatic QTL effects, *b Additive effect of resistance allele, where a positive value indicates that the allele was inherited from the resistant bread wheat parent KK4500, and a negative value indicates that the allele was from the susceptible durum wheat parent Karim.

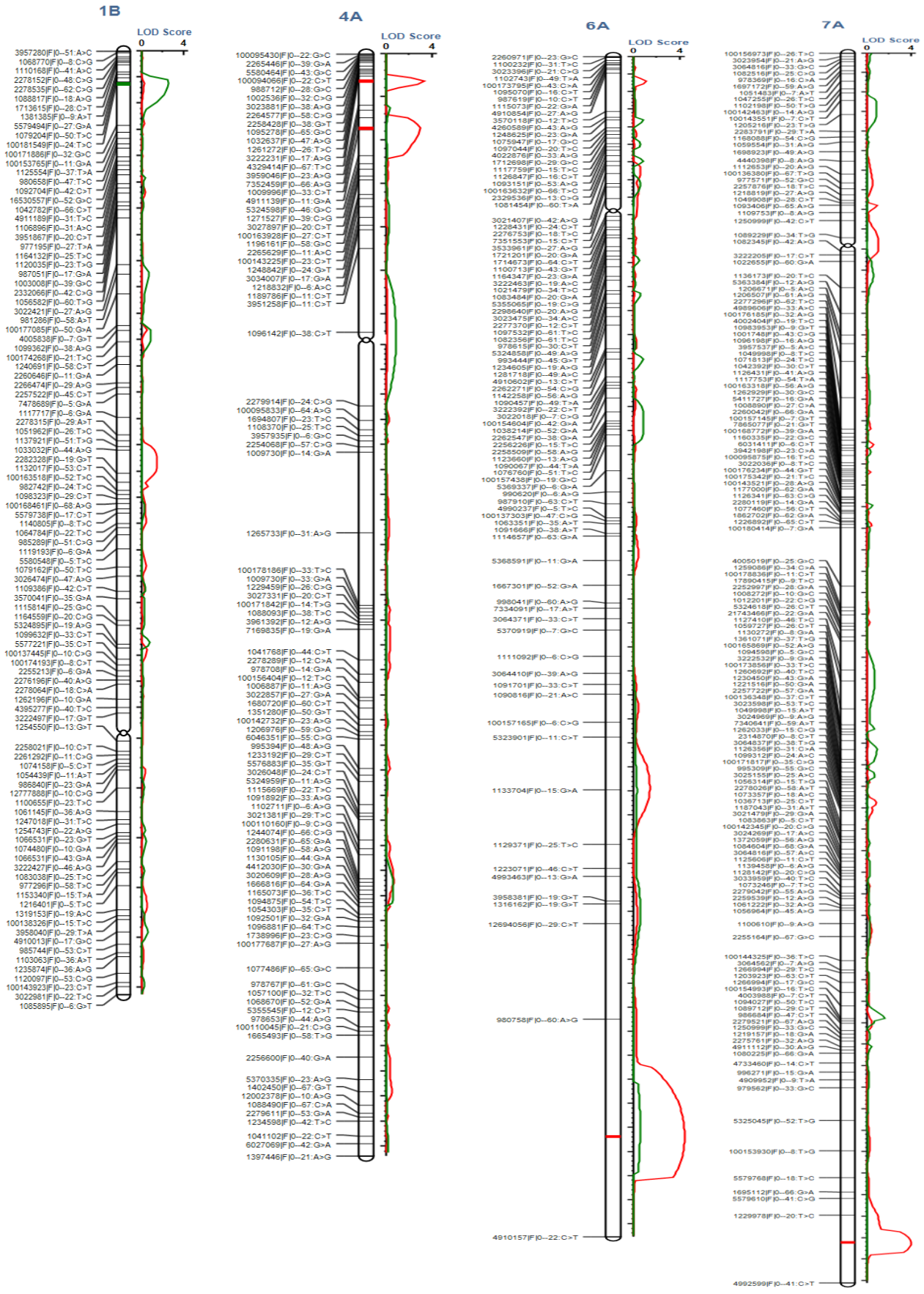

Figure 3-17. QTL related to plant height on the chromosome 1B and to STB disease on the chromosomes 4A, 6A and 7A (Green peacks: plant height, Red peaks: STB disease resistance).

3.4 Discussion

3.4.1 Development of populations

Due to the dimorphic characteristic of the *Z. tritici* pathogen towards wheat species, the aim of this work was to study to effect of *Stb* genes identified in bread wheat and transfer into susceptible durum wheat through pentaploid crosses, against durum wheat specific isolates of *Z. tritici*. Mapping populations between bread and durum wheat offer the opportunity to uncover the genomic region that provides bread wheat with resistance to the durum wheat specific isolates of the pathogen.

With the aim of transferring two *Stb* genes located on the A-genome (*Stb6* and *Stb17*) into two susceptible durum wheats and assessing their effect for *Z. tritici* durum wheat specific strains, six pentaploid populations were developed using three bread wheat genotypes; "KK4500", "SH M3" and "Synthetic 6X" crossed to two susceptible durum wheat genotypes "Karim" and "Om Rabiaa 5". The pentaploid F_1 hybrids were then backcrossed twice to the recurrent durum wheat parent. To obtain the highest number of fertile F_1 progeny from an interspecific cross, it has been proposed that the higher ploidy species should be used as the maternal parent (Kihara, 1982) and this has been the case in most studies to date (Mesfin *et al*., 1999; Lanning *et al.,* 2008; Eberhard *et al.,* 2010; Martin *et al.,* 2011; Kalous *et al.,* 2015). Hence in this study, the bread wheat was used as the female parent to generate the F_1 hybrid seed. In this study, the C7 and C8 populations that share the same bread wheat parent "Synthetic 6X" crossed to the durum wheat genotypes "Karim" and "Om Rabiaa 5" respectively, produced the highest amount of F_1 seed, indicating a high compatibility level between "Synthetic 6X" and both of the durum wheats (**Table 3-6**). The C2 cross combination between "KK4500" and "Om Rabiaa 5" produced the least F_1 seed with the lowest seed set per cross from the pentaploid cross combinations (**Figure 3-18**).

The pentaploid lines (35 chromosomes), were backcrossed to the corresponding durum parent as the female parent. Overall, the seed set per cross improved at every backcross generation to the recurrent parent, with the highest BC_2-F_1 seed produced in the C1 population (**Figure 3-18**). This gradual improvement of seed produced after every round of backcrossing can be explained by the gradual loss of the univalent D-genome chromosomes and the

gradual restoration of the tetraploid level. In fact, cytogenetic analysis, using mc-GISH of 21 randomly selected BC_2-F_1 plants from these crosses (except the C2 population), showed that 70% of the lines had lost all the D-genome chromosomes and the other 30% had a very low retention of D-genome chromosomes that didn't exceed three chromosomes (**Figure 3-4**).

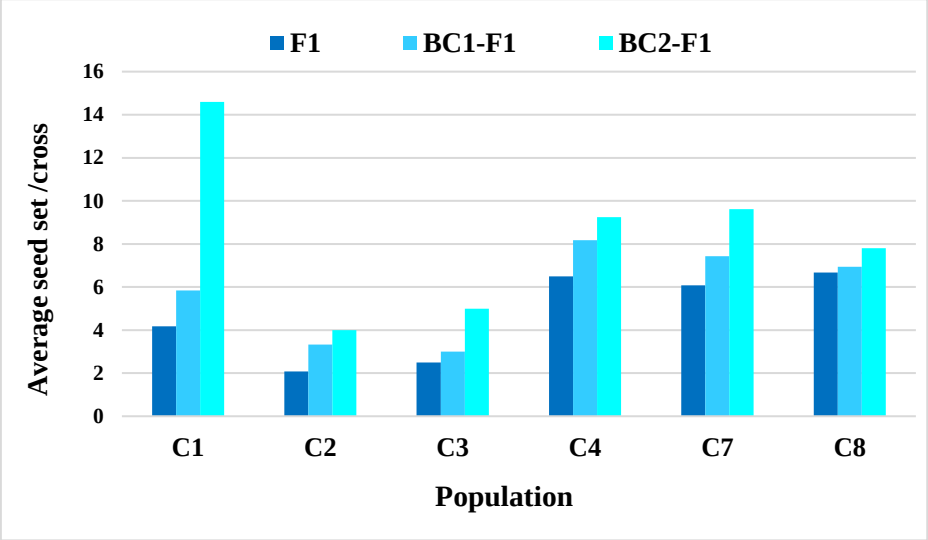

Figure 3-18. Average seed set per cross in the six pentaploid population of the direct bread/durum wheat crosses.

A pentaploid three way crossing strategy was established to study the effect of the *Stb* resistance genes, *Stb5*, *Stb10* and *Stb16*, located on the D-genome of the bread wheat genotypes "Synthetic 6X", "KK4500" and "SH M3" respectively, in durum wheat. To increase the chance of transferring the D-genome chromosomes carrying the corresponding *Stb* genes into durum, LND-DS lines carrying a pair of D-genome chromosomes of the same group as the D-genome chromosome group carrying the *Stb* gene, were crossed to the bread wheat. Using the LND-DS lines as the female parent, viable F_1 seed was produced out of all twelve cross-combinations established (every bread wheat was crossed to two LND-DS lines, where the pair of D-genome chromosome substituted either a pair of A- or a pair of B-genome chromosomes of the same genomic group).

Crosses between the bread wheat genotype "Synthetic 6X" and the LND-DS lines LD7 and LD14 carrying 7D (7A) and 7D (7B) substitutions, respectively, had the highest crossability level (**Figure 3-19**). The lowest seed set was when the LND-DS lines LD1 and LD18 carrying the 1D (1A) and 1D (1B)

substitutions, respectively, were crossed to the bread wheat genotype "KK4500". Crosses of the bread wheat genotypes to the Langdon disomic D (B) substitution lines showed a higher crossability level than crosses to the Langdon disomic D (A) substitution lines. Hence, variable crossability levels are distinguished when LND-DS lines are crossed to different hexaploid genotypes (**Figure 3-19**). In previous studies it has been shown that the crossability of hexaploid wheat with rye is controlled by the dominant or recessive *Kr* alleles located on chromosomes 5B (*Kr1*), 5A (*Kr2*), 5D (*Kr3*) and 1A (*Kr4*) (Riley and Chapman 1967, Krolow 1970, Fedak and Jui 1982, Luo *et al.,* 1992). In both hexaploid and tetraploid wheat, crossability with rye is facilitated by recessive alleles or inhibited by dominant alleles (Krolow, 1970; Fedak and Jui, 1982). In addition, Deng *et al.* (1999) found that the monosomic 1D (1A), 6D (6A) and 7D (7A) F_1 hybrid substitution lines (obtained by crossing the corresponding LND-DS line to the tetraploid "Ailanmai") had a significantly higher crossability level when crossed to rye (*Secale cereal* L.) than the 2D (2B), 3D (3B) and 4D (4B) lines. This high crossability of the tetraploid "Ailanmai" to rye was attributed to recessive crossability alleles on chromosomes 1A, 6A and 7A of the durum genotype (Liu *et al.,* 1999). Furthermore, differences in the average F_1 seed produced per single crossed head can be noticed between the crosses using different hexaploid parents. For instance, the highest number of crossed seed was obtained using the "Synthetic 6X" genotype. This result implies that the choice of hexaploid parent plays a role in the successful production of F_1 progeny in pentaploid crosses when using the LND-DS lines.

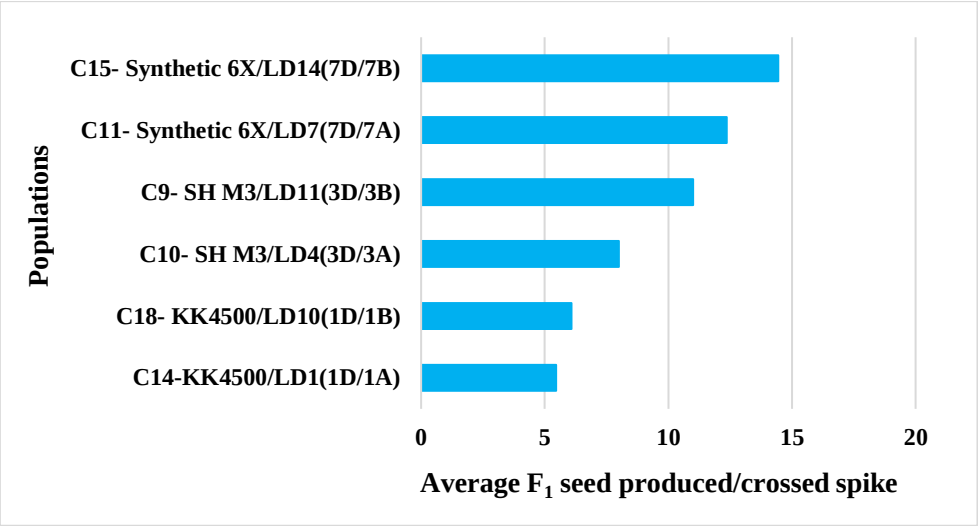

Figure 3-19. Average F_1 seed produced per crossed spike in the pentaploid hexaploid/LND-DS lines crosses.

The F_1 hybrid seed, produced from the six cross-combinations described above, were then top and backcrossed to either of the durum wheat genotypes "Karim and Om Rabiaa 5" to produce the F_1T and the BC_1-F_1 generation, respectively. In these crosses, in contrast to the first crossing strategy, a fluctuation in the average seed set per cross was seen after backcrossing the F_1T generation to the recurrent durum wheat parent (**Figure 3-20**). Some crosses, such as the C9-Karim and C9-Om Rabiaa 5 crosses had a higher BC_1-F_1 average seed produced/cross, whereas the majority of the crosses had a lower BC_1-F_1 seed produced/cross compared to the F_1T seed. This can partially be explained by a thrips (*Haplothrips tritici*) infestation of the glasshouse that affected the seed set and especially that of the crossed spikes. However, cross compatibility between the F_1 and either of the durum parents, as well as the unstable genomic composition of the F_1T lines caused by the use of LND-DS lines to generate the F_1, may have affected their fertility and therefore the seed produced/cross.

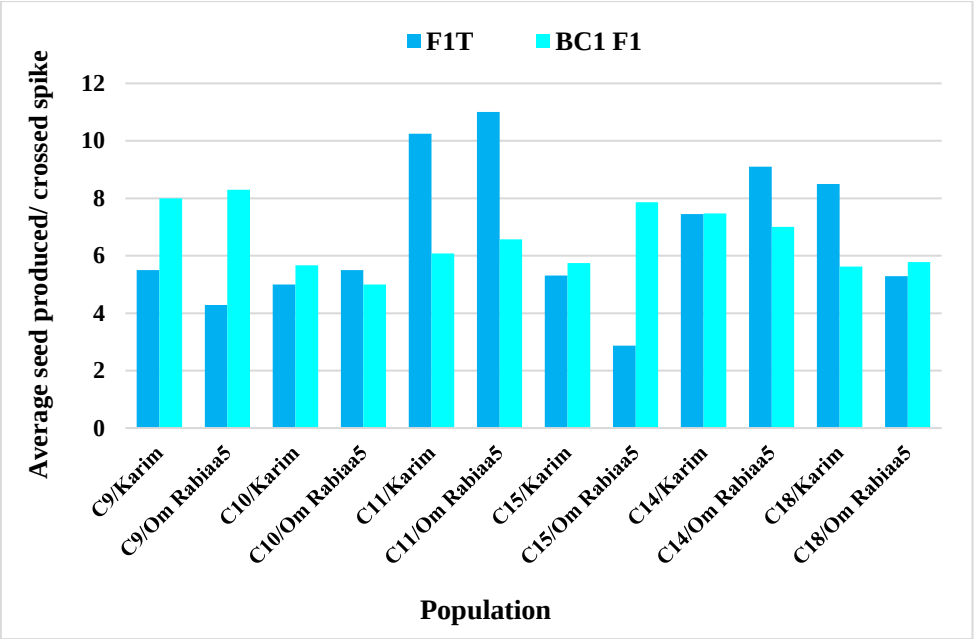

Figure 3-20. Average F_1T and BC_1-F_1 seed produced per crossed spike in the 12 populations of the three way crosses (2^{nd} crossing strategy).

3.4.2 Cytogenetic analysis of D-genome retention in derived lines of pentaploid crosses

Mc-GISH analysis of randomly selected lines from the first crossing strategy (in the BC_1-F_2 and BC_2-F_1 generations) and from the second crossing strategy (in the F_2T and BC_1-F_1 generations) revealed higher average retention rate of the D-genome using the second crossing strategy. As expected, normal genomic inheritance of the A- and the B-genome was seen in the lines from first crossing strategy (presence of the full set, 2n=28, AABB). However, 24% (17 lines out of 70) of the lines screened from the second crossing strategy failed to recuperate the A- or B-genome chromosomes initially substituted in the LND-DS lines used. A higher percentage of lines missing the corresponding A or B-genome chromosomes was distinguished in the BC_1-F_1s (36%) compared to the F_2Ts (11%), suggesting that these chromosomes have a higher chance of becoming homozygous after self-fertilisation compared to backcrossing to the durum wheat parent. All the whole D-genome chromosomes or telomeres retained were present as monosomic additions.

Spontaneous genomic rearrangements involving the D-genome with the A-genome were distinguished in four different lines two of which also contained a telocentric D-genome chromosome. Two of these translocations (D-a and A-d) were found in two BC_1-F_2 lines belonging to the first crossing strategy. The

other two translocations (D-a and A.D) were identified in the progeny of the three way crosses at the F_2T generation. A previous study has demonstrated the occurrence of such translocations in lines derived from pentaploid crosses (Eberhard *et al.,* 2010). Out of 26 lines analysed using a mc-FISH, Eberhard *et al.,* (2010) identified three lines with a single translocation involving the A- with the D-genome in the F_2 generation, of which two were centromeric A.D translocations and one a telomeric A-d translocation. In fact, there is a general tendency for univalent chromosomes to undergo centric breakage-fusion leading to chromosome translocations (Sharma and Gill, 1983). The univalent state of the D-genome chromosomes in the pentaploid crosses promote its breakage and therefore the occurrence of translocations. However these translocations are spontaneous and happen at a low rate. In our case, only 3.2% of the screened lines showed the presence of D-genomic translocations.

3.4.3 The assessment of the effect of the D-genome on STB disease in durum wheat through the phenotyping of LND-DS lines

Several resistance genes for STB disease have been mapped on the A-, B- and D-genomes of bread wheat (reviewed in Brown *et al.,* 2015). Multiple sources of STB resistance have been identified in bread wheat. However, durum wheat, which lacks the D genome, exhibits significant differences from bread wheat in STB resistance even though they share the A- and B-genomes. This might be due in part, to the specificity of isolates of *Z. tritici* in some regions to either of the wheat species (Eyal *et al.,* 1973; Kema *et al.,* 1996a, b; Zhan *et al.,* 2004).

It has been previously speculated that D-genome chromosomes and the durum genetic background may interact with FHB resistance genes and affect their expression (Gilbert *et al.,* 2000). To study the effect of the D-genome and their homoeologous A/B-genome chromosomes on STB disease in durum wheat, the set of 14 LND-DS lines (Joppa and Williams, 1988) were phenotyped at both the seedling and adult stages in both controlled conditions and field conditions, respectively. Phenotyping results revealed a differential reaction to STB disease between both of the growing stages in most of the substitution lines. For the *Z. tritici*-wheat pathosystem, cultivar-by-isolate interactions are known. However, adult-plant responses do not necessarily reflect the responses

of seedlings to the pathogen (Kema and van Silfhout, 1997; Chartrain *et al.,* 2004a). *Stb17* is an example of a gene with a quantitative effect on disease which is expressed in adult plants but not seedlings (Ghaffary *et al.,* 2012). "Langdon" showed a good level of resistance with a mean percentage of SDS that didn't exceed 30% at either the seedling or adult stages.

Some of the substitution lines had an even lower mean percentage SDS at the seedling stage such as substitution lines 2D (2A), 5D (5A), 1D (1B) and 4D (4B). However at the adult stage, field based phenotyping revealed that only the 7D (7B) substitution line had a very low SDS mean score which was lower than "Langdon". Looking at the LND-DS lines that carry the same pair of D-genome chromosomes substituted for either a pair of A- or B-genome chromosomes of the same linkage group, there was no clear relationship between the presence of a particular D-genome chromosome and the improvement of STB resistance. Thus the observed enhanced resistance in some of these lines was mainly related to the A or the B-genomes. For instance, in comparison to "Langdon", the SDS mean disease score increased at the adult stage in LD-7, LD-10 and LD-11, carrying the 7D (7A), 1D (1B) and 3D (3B) substitutions, respectively. Whereas at the seedling stage, the SDS mean disease score increased in LD-4, LD-7 and LD-14, carrying the 3D (3A), 7D (7A) and 7D (7B) substitutions respectively. Such results suggests that chromosomes 7A, 1B and 3B may contain genes that enhance STB disease resistance at the adult stage, whereas chromosomes 3A, 7A and 7B may harbour resistance genes that are only expressed at the seedling stage. This indicates that resistance in the "Langdon" genotype is mutagenic with an additive effect. It has been previously demonstrated in field trials, that resistance to STB generally appears as a quantitative trait, largely additive in nature with some dominance, controlled by an oligogenic or polygenic system with moderate to high heritability in both durum wheat (van Ginkel and Scharen, 1987, 1988; Berraies *et al.,* 2014a) and bread wheat (Danon and Eyal, 1990; Jlibene *et al.,* 1994; Simon *et al.,* 1998).

The presence of the 4D chromosomes in both substitution lines LD-5 and LD-12 increased the mean SDS score at the adult stage. Hence, the 4D chromosome of "CS" cv. might contain genes for STB susceptibility and/or suppression of resistance. In a similar study of the effects of D-genome chromosomes on FHB resistance in durum wheat using the LND-DS lines, Zhu

et al. (2016) didn't find any specific D-genome chromosome that could enhance FHB disease resistance and again the resistance was more related to chromosome 2B and possibly chromosomes 6A and 6B that may contain genes that enhance FHB resistance. They also found that "CS" chromosome 6D might contain genes for FHB susceptibility and/or suppression of FHB resistance (Zhu *et al.,* 2016). When the 14 LND-DS lines were tested for stem rust race 15B-1 and leaf rust race 15, in comparison to "Langdon" which was resistant to both of the diseases races, several substitution lines were found to be more susceptible than "Langdon" (Bai and Knott, 1992). Chromosomes 1B, 2B, and 7B were found to carry resistance genes to stem rust, while chromosomes 2B and 4B carried genes for resistance to leaf rust, and 1D and 3D carried resistance suppressor genes (Bai and Knott, 1992).

This differential reaction of the LND-DS lines for STB disease resistance at both stages could possibly be explained by a genetic variation between this lines, which can be the result of not using exactly the same Langdon genotype in the crosses while developing these set of 14 substitution lines. Taken a closer look to the GBS data available, a very low SNPs and heterozygous call were found between for the 14 LND-DS lines for the same chromosomes e.g. 1A. Hence, this denies the hypothesis that the impurity of these lines in terms of the Langdon background used could have affected the variation of the phenotypic variation observed for STB disease.

The set of LND-DS lines have played an important role in the characterization of durum A- and B-genomes (Joppa and Williams, 1988; Joppa, 1993; Watanabe *et al.,* 1994; Li *et al.,* 2006; Klindworth and Xu, 2008). The use of the LND-DS lines for the screening of disease resistance, has the potential to determine either the loss of a gene(s) on the A or B chromosome which was replaced by the substituted D-genome chromosome, or the identification of a suppressor/resistant gene(s) on the substituted D-genome chromosome.

3.4.4 Populations phenotyping to STB disease under field conditions

Part of the F_1 pentaploid seed produced from the two crossing strategies, as well as the BC_1-F_1 of the first crossing strategy and the F_1T generation of the

second crossing strategy, were phenotyped in field conditions under a high level of disease pressure on the experimental platform located in the north of Tunisia. This region is known to be a *Z. tritici* hot spot with environmental conditions conducive for disease progress during the season. Three liquid inoculations, using five aggressive isolates originating from the same region, were applied before stem elongation to ensure a good infection level. Bread wheat is completely immune in this region to STB disease and this region is therefore known to harbour durum wheat specific strains of *Z. tritici*. All of the pentaploid F_1 hybrid plants phenotyped (12 cross-combinations) were completely immune and didn't show any disease symptoms. This result indicates that the resistance present in the bread wheat genotype is dominant. This is in agreement with a previous study where pentaploid crosses showed an improved resistance to FHB compared to the susceptible durum parent (Gilbert *et al.*, 2000).

Despite the small amount of BC_1-F_1 and F_1T seed produced, seeds of most of the populations were screened in the Septoria platform in Tunisia (except for the C10-K and C10-O populations) Segregation for STB disease resistance was distinguished in all of the screened populations, with 16% and 22% completely resistant (R) plants from the first and the second crossing strategies, respectively. The rest of the plants were moderately resistant (MR) with SDS mean scores that didn't exceed the 50%. None of the plants was seen to be as susceptible as the susceptible durum wheat parent. The highest number of R plants were from the F_1T-C14-O (KK4500/LD-1/2/Om Rabiaa 5), F_1T-C15-K (Synthetic 6X/LD-14/2/Karim) and F_1T-C15-O (Synthetic 6X/LD-14/2/Om Rabiaa 5) populations (**Table 3-14**). These three populations were produced using LD-1 or LD-14, which were shown to be resistant to STB disease at the adult stage (**Figure 3-9**) and can at least partly explain the high number of resistant plants found in these crosses.

The presence of MR plants in the BC_1-F_1 and F_1T generations, after backcrossing of the F_1 to the durum wheat parent, indicates that the resistance might be multigenic with additive effect. These genes are potentially those responsible for the immune reaction of bread wheat to the durum wheat specific *Z. tritici* isolates in the northern region of Tunisia and hence their introgression into durum wheat has enhanced its level of resistance.

The pentaploid crosses in this study have proved to be an efficient way to transfer disease resistance from bread to durum wheat for STB disease. The use of pentaploid crosses has been successfully used previously to transfer disease resistance between bread and durum wheat. For instance, Martin *et al.* (2013) successfully transferred partial resistance to crown rot disease from the bread wheat genotypes "Sunco" and "2-49" into durum wheat while Gilbert *et al.* (2000) were able to improve durum wheat resistance to FHB.

3.4.5 Study of the effect of the *Stb* resistance genes on STB disease resistance in the genomic background of two susceptible durum wheat cultivars

The 665 plants phenotyped on the Tunisian Septoria platform were screened for the retention of the *Stb* genes using closely linked SSR markers (depending of the *Stb* genes present in the parental bread wheat used) (**Table 3-1**). The closely linked *Stb7*/*12* genes were found to be present in both susceptible durum wheat parents. Thus, these two genes, previously mapped on chromosome 4AL in the CIMMYT bread wheat line "KK4500" (Chartrain *et al.*, 2005a) and known to be isolate-specific in bread wheat, are not functional in durum wheat for durum wheat specific isolates of *Z. tritici*. These genes might have been introgressed into wheat during the early stages of wheat domestication. The expression of these genes might be specific to certain *Z. tritici* isolates. Makhdoomi *et al.* (2015) showed that among 420 cultivar × *Z. tritici* isolate interactions, 28% had isolate-specific interactions. In addition, like many other fungal pathogens, the durability of these R genes can be circumvented by adaptation of *Z. tritici* populations to resistance cultivars. This adaptation is known to be correlated with the genetic structure of the pathogen population. *Z. tritici* has a high evolutionary rate, and sexual reproduction is very frequent during the growing season, leading to great genetic diversity that enables the fungus to circumvent monogenic based resistance (McDonald and Martinez 1990a; b).

None of the genes located on the D-genome, *Stb*5 (7DS) or *Stb10* (1Dc) were transferred to durum wheat. In fact, during the backcrossing of the F_1 pentaploid to the recurrent durum wheat parent, most of the univalent D-genome chromosomes will be gradually eliminated resulting in stable tetraploid lines

(Padmanaban *et al.*, 2017a). The use of the LND-DS line to promote and facilitate the retention of the 1D-chromosome carrying *Stb* genes did not work in this crossing programme.

The *Stb17* gene, mapped on chromosome 5AL of "SH M3", was present in 97 % and 24% of the plants screened from the C3 (SH M3/Karim) and C4 (SH M3/Om Rabiaa 5) populations, respectively. This gene was also found to be highly retained (97%) in the C9-K (SH M3/LD-11/2/Karim) population. The high retention of this particular gene was due to the high retention of chromosome 5A from bread wheat in the derived lines of the pentaploid cross. This relatively high retention of chromosomes from linkage group 5 has also been observed in the work with wheat/wild relative introgressions at Nottingham. For example, linkage group 2 of *Aegilops speltoides* was transferred to 100% of the progeny due to the presence of a gametocidal gene but the second most retained linkage group was 5 at 51% (King *et al*., 2018).

Many examples in the literature have shown the effectiveness of some resistance genes when are transferred between bread and durum wheat. One example is *Yr53*, which confers resistance to stripe rust in durum wheat, and was transferred into a susceptible bread wheat genotype through pentaploid crossing (Xu *et al.*, 2013). The progeny derived from the crosses were cytologically selected, based on the presence of all seven pairs of D chromosomes, and were tested with stripe rust race PST100. The progeny of the F_3 generation segregated in a 3:1 resistant: susceptible ratio, suggesting that a single dominant gene was responsible for the resistance (Xu *et al.*, 2013). In another study, Rong *et al.* (2000) transferred the powdery mildew resistance gene *Pm26*, present on chromosome 2BS from a wild emmer accession into bread wheat. In the present study, Chi squared analysis revealed the absence of a significant correlation between the presence of the retained *Stb6* and *Stb17* genes and STB disease severity in the tetraploid background of both susceptible durum wheat genotypes (**Table 3-20**). Hence, it can be concluded that *Stb6* and *Stb17*, which provide partial resistance in bread wheat, are not effective for durum wheat specific isolates of *Z. tritici* and therefore cannot be used in durum wheat breeding to STB disease. It is also interesting to note that both the successful examples above involved transfer of a gene from durum wheat into bread wheat, rather than the transfer direction being attempted in this study.

All of the *Stb* genes identified in bread wheat were mapped using only a limited number of isolates ranging from one (e.g. *Stb17*) to four isolates at most (e.g. *Stb18*) (reviewed in Brown *et al.,* 2015). These genes conferred a partial resistance in bread wheat and are considered to be generally effective against some avirulent isolates but not against virulent of *Z. tritici*. Their pattern of interaction may accords with the gene-for-gene relationship that has only been demonstrated for *Stb6* (Brading *et al.,* 2002). The cloning of this gene revealed that it encodes a conserved wall-associated receptor kinase (WAK)-like protein, which detects the presence of a matching apoplastic effector and confers pathogen resistance without a hypersensitive response (Saintenac *et al.,* 2018). In a study of the effectiveness of the *Stb* genes against Iranian isolates of *Z. tritici*, Makhdoomi *et al.* (2015) found that most of the *Stb* genes are ineffective against the Iranian populations, in particular, *Stb6* and *Stb7*. In addition, the screening of the hexaploid Cvs. "Oasis", "Veranapolis", "Tadinia", "Synthetic 6X", and "Kavkaz-K4500" known to carry *Stb* genes and considered to be sources of resistance to STB in the United States and Europe (Abrinbana *et al.,* 2012), showed that these cultivars have only limited protection against one or a few isolates of *Z. tritici* in Iran (Makhdoomi *et al.,* 2015).

Results of the present study suggest that the ability of the *Z. tritici* pathogen to be cultivar specific (Czembor *et al.,* 2011; Abrinbana *et al.,* 2012; Grieger *et al.,* 2005) and species specific (Zhan *et al.,* 2004) negatively affects the effectiveness of bread wheat resistance genes when introgressed into durum wheat.

3.4.6 Genomic inheritance in population derived from pentaploid crosses

In pentaploid wheat hybrids, the predominance of heterozygous loci present on the A- and B-genomes, together with the retention of a haploid D-genome, results in breeding material that has captured a high degree of genetic variation. Using genotyping by sequencing, the quantification of the genomic proportions of the A-, B- and D-genome alleles inherited from the bread wheat parent in all the lines screened, showed that the mean percentage of the A- and B-genome alleles inherited from the bread wheat parent varied between the different populations (**Table 3-22**). Previous work by Martin *et al.* (2011) also showed

that the relative inheritance of A and B alleles from bread and durum wheat differed among hexaploid/tetraploid crosses. Considering the results of the C3 and C4 populations screened in the BC_1-F_3 generation, that share the same hexaploid parent "SH M3" crossed to either "Karim" or "Om Rabiaa 5" respectively, a higher retention rate of the A and B bread wheat alleles occurred in the C3 population (32%) compared to the C4 population (17%). However, no big difference was seen between the C1 (18%) and C7 (15%) populations screened at the BC_1-F_2 generation and which share the same durum wheat parent "Karim" crossed to either "KK4500" or "Synthetic 6X" hexaploid parent. These results suggest that the durum wheat parent has a major effect on the retention of bread wheat alleles in tetraploid derived lines of pentaploid crosses.

Individual lines from all populations were found to retain a variable amount of bread wheat alleles across individual chromosomes. The low percentage of lines retaining *Stb6* in the C1 (15% of the lines) and C7 (17% of the lines) populations is consistent with the low retention rate of the 3A bread wheat alleles in both of the populations, calculated as 19% and 14%, respectively. In addition, the high retention rate of *Stb17* (located on the 5A) in 97% of the BC_1-F_3-C3 progeny, can be explained by the high retention rate of the 5A bread wheat alleles revealed by DArT markers in this particular population. In fact, the 5A chromosome alleles were found to be the second most retained (50%) after the 3B chromosome alleles (52%) of the bread wheat parent in the C3 population.

Due to the absence of the D-genome in durum wheat and the presence of the *Ph1* gene, no recombination involving the D-genome occurred in the pentaploid crosses. The retention rate of the D-genome chromosomes was expected to be considerably lower after backcrossing of the F_1 pentaploid to the tetraploid parent and this was confirmed via mc-GISH analysis which showed about 68% of the BC_1-F_1 lines had lost all the D-genome. DArT marker analysis also showed a very low retention rate of the D-genome alleles per population (**Table 3-23**). Pearson correlation analysis between STB severity and the mean percentage of the D-genome retained showed the presence of a significant negative correlation (P-value = 0.05, Corr. Coef. = -0.177) in only the C7 population (**Table 3-24**) suggesting the D-genome of the hexaploid genotype "Synthetic 6X" may harbour a partial resistant gene(s) contributing resistance

to the durum wheat specific isolates of *Z. tritici* in a durum background. Due to the difficulty of introgressing a targeted D-genome segment into durum wheat through pentaploid bread/durum wheat crosses, future investigation of the D-genome region conferring this partial STB disease resistance might be better centred on the *Ae. tauschii* accession used to produce the "Synthetic 6X" genotype. Introgression from *Ae. tauschii* into durum wheat could be considered for durum wheat improvement for STB disease resistance. Studies showed that *Ae. tauschii* is an important source of STB resistance genes. May and Lagudah, (1992) have evaluated a worldwide collection of *Ae. tauschii* accessions for their reaction to *Z. tritici* infection using isolates originating from Argentina. Results showed that almost 90% of the accessions evaluated were resistant to *Z. tritici*. In the same study, the first and the second generations of a hybrid population from a selected SH wheat produced by the hybridizing of tetraploid wheats with resistant accessions of *Ae. tauschii* were screened for STB resistance. Two-thirds of the SHs were resistant to STB, which suggested that the D genome of *Ae. tauschii* may carry at least one dominant STB resistance gene (May and Lagudah, 1992). So far, no studies have been conducted to investigate the potential use of *Ae. tauschii* for durum wheat improvement to STB disease.

Pearson correlation analysis showed a significant positive correlation (P-value =0.01, Cor. coef. = 0.983) between the mean percentage of D-genome alleles and A- and B-genome alleles retained from the bread wheat parent. For example, population C3 that retained the highest percentage of A- and B-genome alleles from the bread wheat parent (32%) (**Table 3-22**), was also found to retain the highest mean percentage of D-genome alleles. In previous work, lines with higher levels of durum- derived A- and B-chromosome segments tended to retain fewer D-genome chromosomes (Martin *et al.,* 2011). This implies that the durum wheat used in such pentaploid crosses should be selected with care. For example, in this work using "Om Rabiaa 5", may be more useful to transfer genes from the A- and B-genomes of hexaploid wheat into durum wheats rather than "Karim". Depending on the trait of interest, subsequent backcrossing or self-fertilisation can more rapidly yield elite tetraploid lines that have lost all D genome material and inherited the targeted traits of the original hexaploid parent.

A better understanding of the mechanisms that control both D-genome retention and the proportion of A- and B-genomes derived from the bread wheat parent will assist durum breeders in selecting parental combinations that will favour inheritance of bread wheat alleles in the progeny, leading to an increase in the genetic diversity of the durum wheat genome and therefore allowing the selection of favourable alleles. A number of desirable characters have successfully been transferred between tetraploid and hexaploid wheats (Sharma and Gill, 1983). For instance, improved levels of crown rot disease resistance have been introduced into durum wheats from several hexaploid sources (Martin *et al.*, 2013).

Field based phenotyping for STB disease in naturally infested area allows selection under highly diverse natural populations of the *Z. tritici* pathogen and therefore the identification of resistance genes effective against a wide range of isolates. In the present study, plants with a good level of resistance to STB disease in the BC_1-F_2 and the BC_1-F_3 generations in the absence of the D-genome, were distinguished suggesting the potential presence of genomic regions in the A- and B-genomes of bread wheat that can help improve the level of resistance of durum wheat. In addition, considering the pathogenic dimorphism behaviour of *Z. tritici* toward wheat species, this genomic region may harbour effective resistance genes against *Z. tritici* durum wheat specific isolates which can be exploited in durum wheat improvement. Furthermore, considering the presence of genetic recombination between the A- and B-genomes of bread and durum wheat and its absence in the D-genome, a genetic map of the A- and B-genome can be constructed in the genotyped populations (e.g. Zhang *et al.*, 2012). Hence, in combination with the field based phenotyping data collected on individual lines for the populations in this work, mapping studies on QTL for resistance to STB disease in these populations will be undertaken in the near future for all genotyped populations.

3.4.7 Preliminary QTL analysis of the STB disease resistance in the C1 population

Four minor QTL were detected on the A genome for STB disease resistance in the BC1-F2 generation of the bi-parental KK4500 and Karim population. Known that plant height is one of the traits that can contribute to resistance via

disease escape mechanism, plant height phenotypic data was used to location the plant height QTL and assess if the resistanec is a result of a pleiotropic effect between both traits. Results showed the presence of QTL for plant height on the short arm of chromosome 1B. The two QTLs for STB resistant were inherited from the bread wheat parent were located on the chromosome arm 4AS. The KK4500 bread wheat cultivar was seen previously to harbour the *Stb7* and *Stb12* genes on the chromosome arm 4AL mapped using a bread wheat specific isolates of *Z. tritici* (Chartrain *et al*. 2005a). In the present study instead, using durum wheat specific isolates of the pathogen two QTL were detected on the short arm of the same chromosome. Using the linked SSR markers to these two genes it was seen that these genes are originally present in the durum wheat parent Karim and have no effect of disease resistance in these line. Another two QTL with a minor effect inherited from the susceptible durum parent were detected on the chromosome arms 6AL and 7AL. This result shows that Karim cultivar harbour a resistance gene with a minor effect. The combination of these resistance genes together has the potential to enhance the level of disease resistance. As previously was seen for wheat-*Z. tritici* pathosystem, the resistance is mainly quantitative and controlled by more than one gene. Several QTLs conferring resistance to STB have been identified in previous studies (Kelm *et al.*, 2012; Risser *et al.*, 2011; Kosellek *et al.*, 2013; Adhikari *et al*., 2015). To date, 167 QTLs of resistance against STB have been detected in a total of nineteen bi-parental mapping populations (Brown *et al.*, 2015). All chromosomes, except 5D, carry at least one QTL or meta-QTL for STB resistance. Only a few studies on the inheritance of STB in durum wheat have been reported (Ferjaoui *et al.*, 2011; Berraies *et al.*, 2013a). The study of inheritance of STB resistance in durum wheat through a cross made between the resistant cv. “Salim” and the susceptible “Karim” showed that the resistance was quantitative, controlled by several genes with minor effects (Berraies *et al.*, 2013a). Future work can include a fine mapping of the resistance gene and for a potential use in the future for resistance improvement of durum wheat to STB disease. SNP markers linked to resistance gene can be converted into KASP markers for an easy use in marker assed selection and the incorporation of the genes into elite durum wheat.

Conclusion

1. Regarding the first hypothesis in the present chapter, the transfer of the *Stb* gene present in the bread wheat didn't confer resistance to the durum wheat to STB disease.
2. In the second hypothesis, the use of LND-DS line have the potential of facilitating the retention of the D-genome harbouring *Stb* genes into durum wheat. However a tracking of the genes in question using molecular markers between generations seems to be necessary for a successful transfer.
3. The study of the genomic inheritance of the A- and the B-genomes of bread wheat and the retention of the D-genome in advanced generations of pentaploid populations backcrossed to durum wheat showed the importance of the parental choice. Certain combination have the potential to retain higher percentage of bread wheat alleles in the durum wheat background. Hence, bread wheat can be a source of genetic variation for durum wheat via pentaploid crosses.
4. The QTL analysis of the C1 population showed that resistance can be transferred from bread wheat into durum wheat. The resistance was seen to be quantitative controlled by a minor QTL.

4 Chapter IV. *Ae. tauschii* introgression into durum wheat

4.1 Introduction

Modern wheats (bread wheat and durum wheat) are allopolyploid species resulting from hybridizations between wild diploid species having the A-, B-, or D-genomes and from natural and man-made selections on the various genotypes. Bread wheat with the chromosome constitution AABBDD is a much-improved species, characterized by the highest productivity among the whole *Triticum* genus. The addition of the D-genome has conferred on this species baking characteristics as well as a wide climatic adaptation compared to durum wheat (AABB) (Zohary *et al.*, 1969). Genetic diversity in *Triticum* species is considered to be a key asset in wheat breeding. The goatgrass species *Ae. tauschii spp tauschii*, recognised to be the D-genome donor of the bread wheat D-genome (Kihara, 1944; McFadden and Sears, 1946), has higher genetic diversity than that of the D-genome of the bread wheat cultivars and landraces

(Reif *et al.,* 2005) and constitutes an important source of useful genes for wheat improvement. This wide genetic variation in *Ae. tauschii* has been mainly exploited for bread wheat improvement (Ogbonnaya *et al.,* 2003). However, the exploitation of *Ae. tauschii* for durum wheat improvement is very limited.

4.1.1 Wheat gene pool

Harlan and de Wet (1971) proposed the concept of three gene pools, primary, secondary, and tertiary, based on the evolutionary distance between the species, the success rate of hybridisation among species, and the feasibility of crossing among them (**Figure 4-1**). The primary gene pool species include the *Triticum* hexaploid landraces, the cultivated tetraploids, wild *T. dicoccoides*, and diploid donors of the A- and D-genomes to durum and bread wheats (Qi *et al.,* 2007). Gene transfers from these species is considered to be relatively easy through standard breeding method such as homologous hybridisation either through direct crosses of these species with bread wheat or the production of synthetic wheat (SH) (McFadden and Sears, 1946; Gill and Raupp, 1987). No special cytogenetic manipulation, except embryo rescue, is necessary to produce F_1 hybrids within this gene pool.

The secondary gene pool is formed of the polyploid *Triticum* plus *Aegilops* species which share one genome with the three genomes (A, B, and D) of wheat. Gene transfers from this gene pool require cytogenetic manipulations to enhance the recombination between alien and wheat homoeologous chromosomes. Wild relatives with genomes that are non-homologous to wheat reside in the tertiary gene pool that includes diploid and polyploid species of *Triticeae*-carrying genomes other than A, B and D. This pool is represented by several species from *Agropyron, Thinopyrum, Secale* and *Elymus* (Harlan and deWet, 1971). Chromosome pairing and recombination in common wheat are largely governed by the gene *Ph1* located on the long arm of chromosome 5B (Riley and Chapman, 1958), and thus special techniques for successful crosses need to be employed for utilization of these species in introgression programmes.

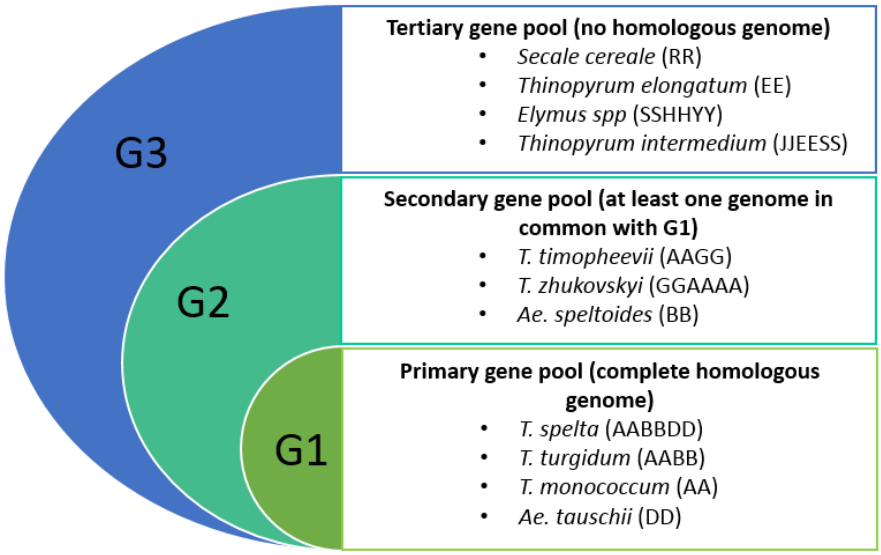

Figure 4-1. Wheat gene pool representing various species (described in Chaudhary *et al.*, 2015).

4.1.2 *Ae. tauschii spp.*

4.1.2.1 Distribution

Aegilops tauschii is a diploid self-pollinating goatgrass species (2n=14, DD) considered to be the D-genome donner of wheat (Dvořák *et al.*, 1998). This species is widely distributed from northern Syria and Turkey to western China in central Eurasia (Van Slageren, 1994), adapted to a variety of environments such as desert margins, steppe regions, stony hills, wastelands, roadsides, sandy shores, and even humid temperate forests (van Slageren, 1994). It is also found at the edges of wheat fields in eastern Turkey, Iraq, Iran, Pakistan, India (Kashmir), China (the Himalaya), Afghanistan, most of central Asia, Transcaucasia, and the Caucasus region (Feldman, 2001). Traditional durum wheat cultivation associated with weedy *Ae. tauschii* was observed at the Alamut and Deylaman-Barrehsar districts of the central Alborz Mountain region in Iran (Matsuoka *et al.*, 2008). *T. turgidum-Ae. tauschii* association, hypothesized in the theory of bread wheat evolution, still exists in the area where bread wheat probably evolved (Matsuoka *et al.*, 2008).

4.1.2.2 Classification

Based on morphology, taxonomists have divided *Ae. tauschii* into two subspecies: *ssp*. *tauschii* and ssp. *strangulata* (Eig) Tzvel. (Eig, 1929; Hammer, 1980). *Ssp*. *tauschii* is further divided into three morphological groups: *anathera*, *meyeri*, and *typica*, whereas *ssp*. *strangulata* is monotypic. The *ssp*.

strangulata is regarded to be the direct donor of D-genome of bread wheat (Nishikawa *et al.,* 1980; Dvořák *et al.,* 1998; Pestsova *et al.,* 2000). Subspecies *tauschii* are characterised by elongated cylindrical spikelets and a distribution from eastern Turkey to China and Pakistan, whereas *ssp*. *strangulate* are characterised by spikes bearing quadrate spikelets and have been found in two disjoined regions, southeastern Caspian Iran and Transcaucasia (Kihara *et al.,* 1965; Yen *et al.,* 1984; van Slagern, 1994; Jaaska, 1995). This classification is controversial because of the existence of morphologically intermediate types (Van Slageren, 1994; Dudnikov, 1998; Matsuoka *et al.,* 2009). Genetic classification based on SNP markers analysis revealed two lineages of the *Ae. tauschii* genepool, designated lineage 1 (L1) and lineage 2 (L2) (Mizuno *et al.,* 2010; Wang *et al.,* 2013). These two lineages are further subdivided into two sub lineages L1W, L1E and L2W, L2E respectively. Results revealed that L1W is found in eastern Turkey, Armenia, Azerbaijan, and western Iran. L1E is distributed from central Iran to China. L2W is located in Armenia and Azerbaijan, and L2E in Caspian Azerbaijan and Caspian Iran (Wang *et al.,* 2013). On the basis of the SNP data, the population within L2E in the southwestern and southern Caspian was shown to be the main source of the wheat D-genome, whereas L1 contributed as little as 0.8 % to the wheat D-genome.

4.1.2.3 Genetic diversity

Allelic diversity in the wild grass *Ae. tauschii* is greater than that in the D-genome of bread wheat and landraces of which *Ae. tauschii* is source (Reif *et al.,* 2005; Cox *et al.,* 2017). The diversity centre of *Ae. tauschii* is considered to be present in the western habitats, from where it probably expanded to the eastern habitats (Matsuoka *et al.,* 2008). The diversity of *Ae. tauschii* has been studied using different molecular tools such as chloroplast DNA variation (Matsuoka *et al.,* 2008, 2009; Takumi *et al.,* 2009), AFLP (Mizuno *et al.,* 2010), SSR (Naghavi and Mardi, 2010), isozymes (Dudnikov and Kawahara, 2006), and RAPD markers (Okuno *et al.,* 1998). Results showed that the genetic diversity of *Ae. tauschii* is much larger than that of the D-genome of *T. aestivum*. However, diversity in wheat's D-genome is also lower than in the A- and B-genomes (Chapman *et al.,* 2015). Diversity analysis using 4,449 polymorphic

DArT markers showed that the diversity of *ssp. strangulata*, comprises only a limited part of the overall diversity of *Ae. tauschii* (Sohail *et al.,* 2012).

4.1.3 Source of useful genes

The extensive screening of *Ae. tauschii* germplasm collections for phenotypic traits with the strongest focus on resistance to fungal pathogens and insect pests, revealed the importance of this species as a source of useful genes for wheat improvement. Several resistance genes to the most damaging pest and diseases of wheat have been identified (Gill *et al*., 1986; Cox *et al*., 1990) such as Green bug, Hessian fly (EL Bouhssini *et al.,* 2013), soil-borne mosaic virus, Powdery mildew (Lutz *et al.,* 1995; Miranda *et al.,* 2006, 2007), Septoria tritici blotch (Arraiano *et al.,* 2001; Simon *et al.,* 2001), Stripe rust (Singh *et al.,* 2000) and Leaf rust (Hiebert *et al.,* 2007). Extensive allelic variations in both seed storage protein composition and isozymes have been detected in *Ae. tauschii* (Lagudah *et al.,* 1987; Lagudah and Halloran, 1989; Gianibelli *et al.,* 2001; Yan *et al.,* 2003a; b).

Furthermore, other genes related to physiological traits (Le *et al.,* 1986; Limin and Fowler, 1981) as well as abiotic stress such as cold (Limin and Fowler, 1981) and salt tolerance (Schachtman *et al.,* 1992) have been identified. In examining *Ae. tauschii* genome sequence, Jia *et al.* (2013) found 1219 protein-coding genes potentially involved in disease resistance, 485 potentially involved in abiotic stress tolerance, 216 potentially involved in cold tolerance and 14 transcription factors associated with drought-tolerance genes. This result highlights the importance of the exploitation of this species in wheat breeding.

4.1.4 Introgression approaches of *Ae. tauschii* into bread wheat

4.1.4.1 Development of synthetic hexaploid wheat

The recreation of spring hexaploid wheat to produce SHW involves crossing tetraploid wheats with *Ae tauschii* and then doubling the triploid chromosome set (ABD) by colchicine treatment or via spontaneous chromosome doubling arising from unreduced gamete formation. SHW that combines genes from *T. turgidum* and *Ae. tauschii*, are arguably the most widely exploited wheat genetic resources as sources of new variation for the improvement of bread wheat (McIntyre *et al*., 2014). This approach has the potential not only to incorporate useful genes from *Ae. tauschii*, but also the tetraploid parent thus enhancing the

A-, B-, and D-genomes simultaneously. Therefore, SHW populations, generated using elite durum wheats and numerous accessions of *Ae. tauschii,* became the storehouse of D-genome allelic diversity that made available a wide range of novel genes for bread wheat improvement encompassing a wide array of target objectives (Mujeeb-Kazi *et al.,* 2008).

4.1.4.2 Direct hybridisation

This approach consists of a direct cross of *Ae. tauschii* with a selected bread wheat genotype. Genetic transfer of the *Ae. tauschii* genome occurs as a consequence of a direct hybridisation and homologous recombination with the D-genome of the bread wheat. The F_1 created (ABDD) is backcrossed to the bread wheat parent to recover a stable bread wheat derivative (Gill and Raupp, 1987). The resulting BC_1 population segregates for chromosome number. Stable 42-chromosome (AABBDD) progeny can then be obtained through self-fertilisation or repeated backcrosses (Cox *et al.,* 2017). The advantage of the direct hybridization approach is to rapidly restore the recurrent parent's A- and B-genomes and avoids incorporation of genes with adverse effects on threshability, hybrid necrosis, vernalisation response, milling and baking quality, and other traits, which are often transferred when *T. turgidum* is used as a parent (Cox *et al.,* 2017).

4.1.4.3 Use of *T. aestivum-Ae. tauschii* substitution lines

A set of seven *T. aestivum-Ae. tauschii* substitution lines were developed by Law and Worland (1973) through the crossing of "Chinese Spring" (CS) to the SHW 'Synthetic 6x', obtained from a cross of the tetraploid emmer and *Ae. tauschii* (McFadden and Sears, 1947). The substitution lines contained a single D-genome chromosome of *Ae. tauschii* that replaced its homologous chromosome in CS. These lines were used to generate a set of well-characterized *T. aestivum-Ae. tauschii* introgression lines (Pestsova *et al.,* 2001; 2006). In fact, the substitution lines were backcrossed twice to CS and the progeny were analysed with 65 SSR markers previously mapped on the D-genome (Roder *et al.,* 1998), in order to select a set of homozygous introgression lines representing the whole *Ae. tauschii* genome. In total, 259 BC_1-F_2 and 450 BC_2-F_2 plants were genotyped and recombinant lines carrying different segments of *Ae. tauschii* chromosomes were detected. Plants

containing small introgressions of the alien genetic material were self-fertilised to get homozygous lines and plants carrying large pieces of the donor chromosome were backcrossed again to get smaller introgressions. In total, 84 different homozygous introgression lines were developed from BC_1 and BC_2 progenies (Pestsova *et al.*, 2001; 2006).

4.1.5 Introgression approaches of *Ae. tauschii* into durum wheat

The introgression *Ae. tauschii* into durum wheat is more complex compared to bread wheat. In this case, cytogenetic manipulation is required to enhance the recombination and induce homoeologous chromosome pairing between the D-genome of *Ae. tauschii* and the A- and the B-genome of the durum wheat. Hence, for successful introgression, two types of durum wheat genotypes can be used for this purpose, the first being the LND 5D (5B) disomic substitution line (Joppa and Williams, 1988) and the second consists of *ph1c* mutant durum lines (Giorgi, 1978, 1983). These two genotypes have been extensively used for alien introgression into durum wheat.

Two *ph1c* mutant durum wheat cultivars: “Cappelli” and “Creso” obtained by seed treatment (Giorgi, 1978, 1983), have been generally used as tools to induce homoeologous pairing in wheat-alien hybrid combinations and targeted alien transfers into durum wheat. In addition, with good compensation of chromosome 5D for 5B, the use of the 5D (5B) disomic substitution line turned out to be an efficient system to promote pairing in a number of interspecific hybrids involving durum wheat and different alien species such as *Thinopyrum spp*. carrying desirable genes for resistance to wheat rusts, barley yellow dwarf virus and fusarium head blight (Jauhar and Almouslem, 1998). In the absence of 5B, chromosome pairing increased more than fourfold in the ABJ triploid hybrids involving the diploid *Th. bessarabicum* (2n=2x=14, JJ) as compared to their counterparts with a normal durum wheat.

In a comparison between durum wheat haploids either carrying the mutated *ph1c* allele or chromosome substitution 5D in place of 5B, intergenomic pairing revealed by GISH, seemed to be higher when using the substitution line than the *ph1c* genotypes (Jauhar *et al.,* 1999). The observed difference in the amount of pairing promotion might be at least partly attributed to the effect of chromosome 5D, which carries a pairing promoter on its long arm, with an

overall effect, at least in *T. aestivum* (Sears, 1976), estimated to be greater than that on the 5BS arm. Chromosome 5D is lacking in the substitution line but present in the *ph1c* mutant.

To date, only a set of durum wheat/*Ae. tauschii* monosomic addition lines have been developed by Dhaliwal *et al.* (1990). This set comprises four normal D chromosomes 1D, 2D 3D and 6D and three translocation chromosomes 4DS-5DS, 5DL-7DS, and 7DL-4DL and were used to produce all 14 chromosome arms of the D-genome as monosomic additions. These lines were produced through the use of a synthetic amphidiploid between the durum genotype 'PBW114' and an *Ae. tauschii* (DD) accession 3754, obtained by spontaneous doubling of the F_1 hybrid. The amphidiploid was then backcrossed to the durum parent several times. Despite the high fertility of these monosomic addition plants, data of Makino (1981) on low gametic transmission of *Ae. tauschii* chromosomes from monosomic addition lines indicated some difficulty in the feasibility of their maintenance or the isolation of the corresponding disomic additions.

4.1.6 Application of *Ae. tauschii* in wheat breeding

Ae. tauschii encompasses a wide range of resistances/tolerances to biotic/abiotic stresses (Valkoun *et al.*, 1990; Cox *et al.*, 1991). Using either of the introgression approaches explained above, several traits of interest have been successfully introgressed into bread wheat cultivars (reviewed in Cox *et al.*, 2017). These traits include genes of resistance to several diseases (Olson *et al.*, 2013; Mandeep *et al.*, 2010; Leonova *et al.*, 2007; Miranda *et al.*, 2006; Ma *et al.*, 1993; Eastwood *et al.*, 1994), bread-making quality (Li *et al.*, 2007), pre-harvest sprouting tolerance (Gatford *et al.*, 2002; Imtiaz *et al.*, 2008) and yield related traits (Gororo *et al.*, 2002). In fact, yield related traits such as kernel size, shape and thousand kernel weight (TKW) have been identified in a genetic wide association study (GWAS) of a collection of SHW conducted by Rasheed *et al.* (2014), where two important loci on 3D and 6D chromosomes were consistently associated with kernel length, width and TKW. Similar observations were made in derived synthetic hexaploid lines where higher yielding lines were associated with increased rates of kernel size and higher TKW, in particular in lower-yielding environments under drought stress (del Blanco *et al.*, 2000; Gororo *et*

al., 2002). The introgression of yield related genes contribute effectively in enhancing the yield potential in wheat.

Something to consider, when introgressing the *Ae. tauschii* D-genome into durum wheat is the presence of two puroindoline genes; *Puroindoline a* and *Puroindoline b* (*Pina* and *Pinb*, respectively), which are coded at the *Hardness* (*Ha*) locus on the distal portion of chromosome 5DS (reviewed in Morris, 2002; Bhave and Morris, 2008a; b). These genes code for the wheat physical texture and condition its end-use properties. In durum wheat completely lacking the *Ha* puroindoline genes, its kernel texture is considered very hard. Several studies reported the successful transfer of *Ha* from *T. aestivum* into the durum wheat genome with the result being a soft kernel phenotype (Gazza *et al.,* 2003; 2008; Simeone *et al.,* 2003, Morris *et al.,* 2011). However, the direct introgression of these genes from *Ae. tauschii* hasn't been reported yet.

The direct introgression of *Ae. tauschii* into durum wheat has not been used to any great extent, with only a few targeted D-genome segments bearing genes of interest being transferred from bread wheat into durum wheat (more in chapter 5). This is possibly due to difficult recombination of *Ae. tauschii* in a cross with the durum wheat compared with bread wheat that already carries the full set of the D-genome.

The hypothesis discussed in this chapter relates to the introgression of *Ae. tauschii* genome segments into the durum wheat using the LND 5D (5B) substitution line to generate a panel of durum wheats with different D-genome segments that ideally can cover as much as possible of all D-genome linkage groups.

4.2 Material and methods

4.2.1 Plant material

The accession "P99-95.1-1" of *Ae. tauschii* (obtained from the USDA gene bank), LND 5D (5B) substitution line and the durum wheat variety "Om Rabiaa 5" were used in the crossing scheme. Seeds were sterilised before germination. The seedling plants were vernalised then potted out in glasshouse conditions and grown until adult stage, as described previously in Chapter 2. The parental

lines were planted at two weeks intervals on three different dates to ensure that both parents were at the correct stage of crossing at the same time.

The interest in using *Ae. tauschii* P99-95.1-1 accession stems from its resistance to STB disease of wheat particularly *Z. tritici* durum wheat specific strains. The screening of this accession in collaboration with the SPPP in Tunisia, for resistance to STB disease at both the seedling and adult stages in controlled and in field conditions confirmed its complete resistance to STB disease. Thus, the choice of the susceptible durum wheat variety "Om Rabiaa 5" to be able to identify the D-segment conferring resistance to STB in the developed *Ae. tauschii*/durum wheat introgression lines in the future.

4.2.2 Crossing plan

Ae. tauschii was used as the pollen donor to cross with the LND 5D (5B) line. The F_1 seed produced were crossed and back-crossed as the female parent to the durum wheat variety "Om Rabiaa 5" (**Figure 4-2**).

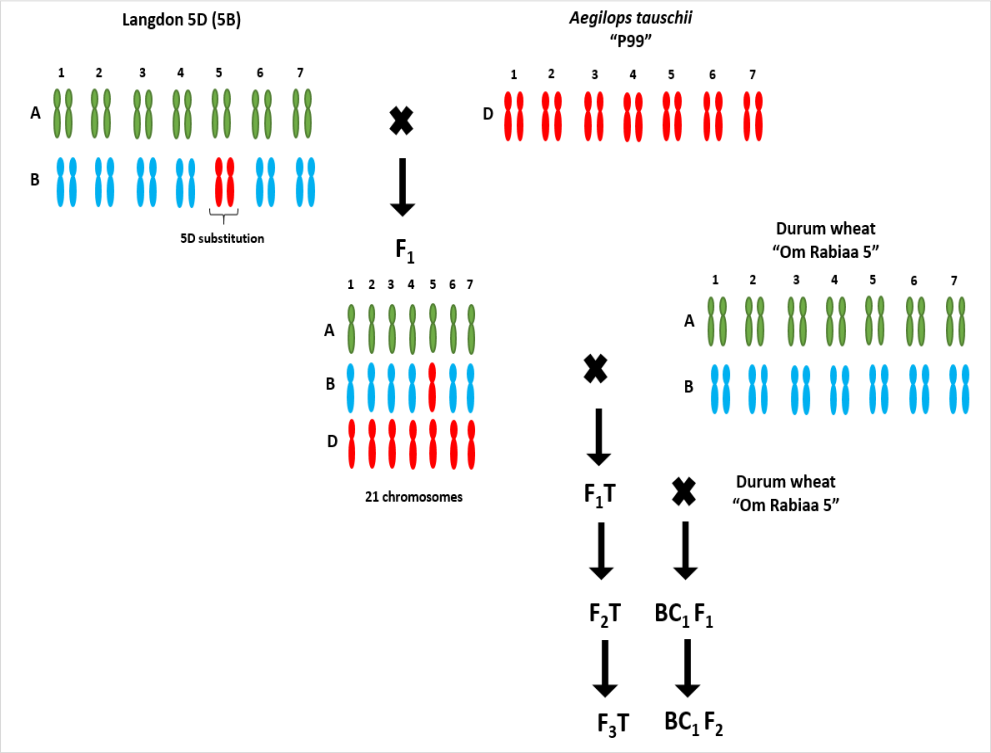

Figure 4-2. The crossing diagram of *Ae. tauschii* introgression into durum wheat.

4.2.3 Cytogenetic analysis of the progeny

4.2.3.1 Multicolour-GISH

Progeny produced from the crossing programme (**Figure 4-2**) were analysed using mc-GISH for the eventual identification of D-genome chromosome

retention or translocation in every generation. For this purpose, root tips were collected straight after seed germination and the mc-GISH protocol applied as described in chapter 2 (section 2.5).

4.2.3.2 Multicolour-FISH

Mc-FISH using the repetitive DNA sequence pA1 of *Ae. tauschii* (Rayburn and Gill, 1986) and the rye clone pSc119.2 (Bedbrook *et al.,* 1980) as probes, was used on the metaphase spreads of the BC_1-F_2 and the F_3T progeny that showed the presence of D-genome chromosome(s) and/or D-genome translocation(s) via mc-GISH. Metaphase spread preparation and the mc-FISH protocol followed are described in Chapter 2 (section 2.6).

4.2.4 Molecular analysis

4.2.4.1 D-genome specific SSR- markers

A set of 21 D-genome specific SSR markers (Appendix 14), with three markers per D-genome chromosome group (on the short arm, close to the centromere and on the long arm), were used to confirm the linkage group of the D-genome chromosome substitution, addition or translocation identified with Mc-FISH analysis in the BC_1 F_3 and the F_3T generation. The PCR protocol followed was as described in chapter 2 (section 2.5).

4.2.4.2 Molecular identification of the *Puroindoline* gene introgression in the 5DS introgression line using STS markers

Screening for the presence of the *Puroindoline* genes; *Puroindoline a* (*Pina-D1*) and *Puroindoline b* (*Pinb-D1*) at the *Hardness* (*Ha*) locus (Bhave and Morris, 2008), in the BC_1-F_2-248-H line using two specific STS markers, showed a single introgression of chromosome arm 5DS. The durum wheat parent "Om Rabiaa 5" was used as a negative control alongside Chinese Spring and the LND 5D (5B) substitution line and *Ae. tauschii* as positive controls. The *Pina-D1*gene was amplified using the forward primer sequence ATG AAG GCC CTC TTC CTCA and the reverse primer sequence TCA CCA GTA ATA GCC AAT AGTG (Gautier *et al.*, 1994). The *Pinb-D1* gene was amplified using forward primer ATG AAG ACC TTA TTC CTC CTA and the reverse primer sequence TCA CCA GTA ATA GCC ACT AGG GAA (Gautier *et al.*, 1994).

DNA extraction and PCR reactions were carried out as described in sections 2.3 and 2.6. DNA samples were denatured at 94°C for 2 min, before 35 cycles

of 45 s denaturation at 94°C, 1 min annealing at 55°C, and 1 min elongation at 72°C, with a final extension of 5 min at 72°C. The PCR products were analysed on 1.5% (w/v) agarose gels, stained with ethidium bromide, and visualized using UV light.

4.3 Results

4.3.1 Seed production

Twenty crosses were made between *Ae. tauschii* and the LND 5D (5B) substitution line, using the alien species as the pollen donor and LND 5D (5B) as the female parent. Out of the 20 crosses made, four amphihaploid F_1 seeds were obtained. However, of these four seeds only one plant successfully grew to reach maturity. The F_1 plant showed a male sterile phenotype, and therefore all the spikes were crossed to the durum wheat genotype "Om Rabiaa 5" as the female parent. At this stage seed produced was designated as F_1 top (F_1T) because of the three different parents involved in the crossing. Only one F_1T seed was produced, germinated and grown to maturity. The F_1T plant was quite weak bearing only four heads. One spike was self-pollinated and the rest were backcrossed to the durum parent "Om Rabiaa 5". Thirteen BC_1-F_1 seed were obtained after backcrossing and two F_2T seeds from the self-pollinated heads. Ten BC_1-F_1 and the two F_2T seeds were germinated. Nine BC_1-F_1 plants grew to maturity and were allowed to self-pollinate to produce the BC_1-F_2 seed (**Table 4-1**). Noticeably, the number of seed set improved in the F_3T and in the BC_1-F_1 and BC_1-F_2 generations after plant backcrossing compared to the F_1T and F_2T generation (**Table 4-1**). Four to six BC_1-F_2 and F_3T seeds from each of the nine BC_1-F_1 lines and the two F_2T for a total of 50 seeds were randomly chosen and germinated. 90% of the seeds (45 out of 50 seeds) germinated and plants reached maturity.

Table 4-1. Number of crosses, self-pollinated heads and seed production in every generation of the introgression program of *Ae. tauschii* into durum wheat.

Generation	Pedigree	Plant reference	No. of crosses	No. of head self-pollinated	No. of seed produced
F_1	LND 5D (5B) /*Ae. tauschii* "P99"	--	20	--	4
F2	LND 5D (5B) /*Ae. tauschii* "P99"	LD6-P99	--	2	0
F_1T	LND 5D (5B) /*Ae. tauschii* "P99"//Om Rabiaa 5	LD6-P99	10	--	1

F_2T	LND 5D (5B) /*Ae. tauschii* "P99"//Om Rabiaa 5	LD6-P99-OR	--	2	2
F_3T	LND 5D (5B) /*Ae. tauschii* "P99"//Om Rabiaa 5	F_2T-254	--	11	26
		F_2T-255	--	8	34
BC_1-F_1	LND 5D (5B) /*Ae. tauschii* "P99"//Om Rabiaa 5*2	LD6-P99-OR	10	--	13
BC_1-F_1	LND 5D (5B) /*Ae. tauschii* "P99"//Om Rabiaa 5*2	BC_1-F_1-244		8	61
		BC_1-F_1-245	--	10	16
		BC_1-F_1-246	--	9	17
		BC_1-F_1-247	--	8	49
		BC_1-F_1-248	--	7	49
		BC_1-F_1-249	--	--	--
		BC_1-F_1-250	--	9	107
		BC_1-F_1-251	--	12	77
		BC_1-F_1-252	--	12	42
		BC_1-F_1-253	--	9	55

NB: --not present

4.3.2 Mc-GISH analysis of the progeny

Progenies produced starting from the F_1 and the F_1T and the following selfing generations until the BC_1-F_3 and F_3T generations were analysed using mc-GISH in a search for genomic translocation involving the D-genome. The notation of the genomic translocations are designated by the letter of the genome involved (A, B or D). An upper case letter designates the largest segment, whereas a slower case letter designates the small segment. In paracentric translocations, the two letters are separated by a dash (e.g. A-d), whereas in centromeric translocations a dot is used (e.g. A.B).

4.3.2.1 Mc-GISH analysis of the F_1 and the F_1T generation

The Langdon 5D (5B) substitution line was confirmed by mc-GISH before crossing to *Ae. tauschii* (**Figure 4-3a**). The mc-GISH of the metaphase spreads of the amphihaploid F_1 plant produced revealed the presence of 21 chromosomes: seven A-genome chromosomes, six B-genome chromosomes and eight D-genome chromosomes (**Figure 4-3b**). After crossing the F_1 plant to the durum wheat parent "Om Rabiaa 5", mc-GISH of the metaphase spread of the F_1T plant showed the presence of eight D-genome chromosomes together

with 14 A-genome chromosomes and 13 B-genome chromosomes (**Figure 4-3c**). The missing B-genome chromosome is probably from the 5B-chromosome group since the 5D (5B) Langdon substitution line was used as the parent. Notably, an A-d translocation was distinguished in the F_1T plant (**Figure 4-3d**). This translocation consisted of a small D-genome segment translocated in the telomeric region of the short arm of an A-genome chromosome.

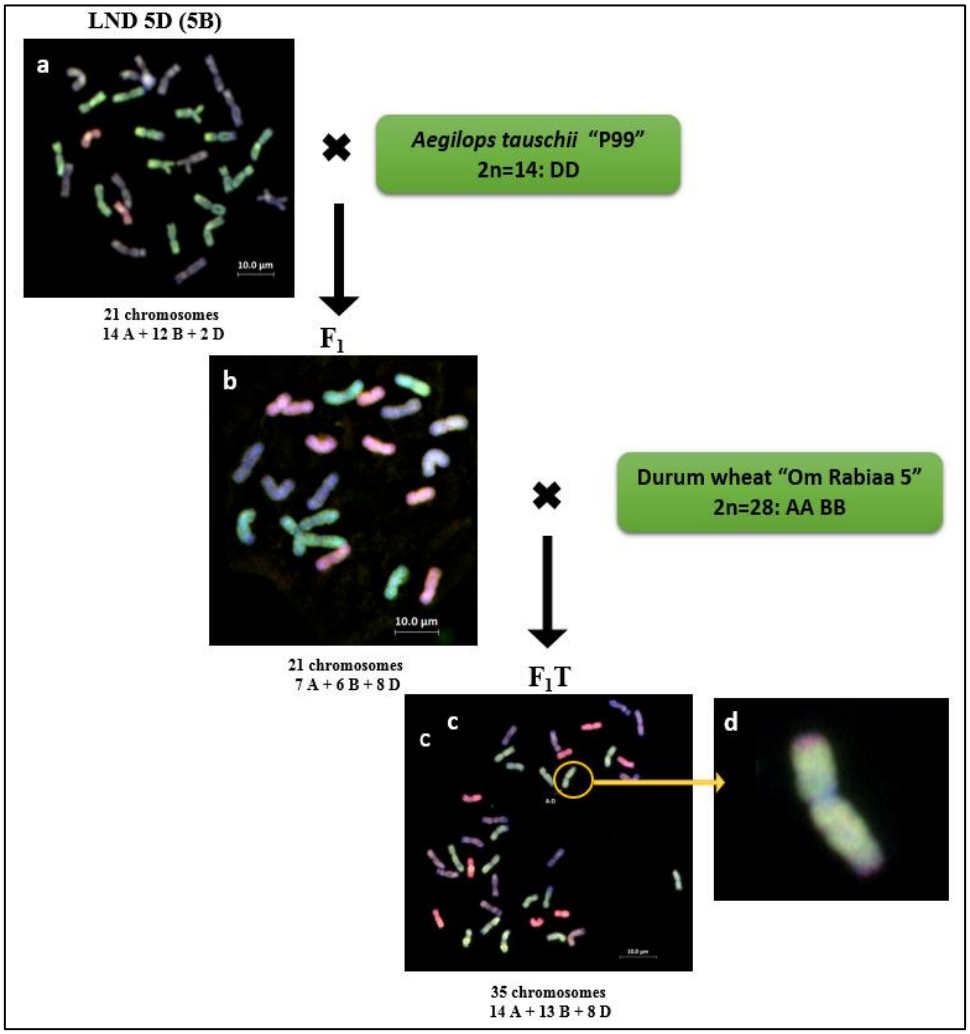

Figure 4-3.Mc-GISH of root-tip metaphase spreads of (a) the LND 5D (5B), (b) the F_1 and (c) F_1T plants in the crossing diagram, revealing the presence of a telomeric A-D chromosome translocation (d) in the F_1T plant (Chromosome colour code: A-genome: green, B-genome: blue/purple, D-genome: red).

4.3.2.2 Mc-GISH analysis of the F_2T and the BC_1-F_1 generation

Cytogenetic analysis of the two F_2T plants and the nine BC_1-F_1 plants, allowed the characterisation of their genomic composition and the identification of genomic translocations involving the D-genome with the A- and the B-genome (**Table 4-2**). All the lines had only 13 B-genome chromosomes with

one B-genome chromosome absent (chromosome 5B). Fifteen A-genome chromosomes were counted in BC_1-F_1-248 suggesting that one of the A-genome chromosome group was transmitted as a pair during meiosis (**Table 4-2**). The D-genome chromosome retention considerably decreased in the BC_1-F_1 after back-crossing to the durum wheat parent, varying between one to three chromosomes (**Table 4-2**). Two BC_1-F_1s carried the telomeric A-d translocation identified in the F_1T plant (e.g. **Figure 4-4a**). A new B.D translocation in the centromeric region characterised as a Robertsonian translocation (RobT) was also identified in one line (**Figure 4-4b**). The two F_2T plants differed considerably in the retention of D-genome chromosomes with one line containing 12 and the other line only two (**Table 4-2**).

Table 4-2.Chromosomal constitution of the BC_1-F_1 progeny and the F_2T lines revealed Mc-GISH.

Gener.	Lines	A-chrom. No.	B-chrom. No.	D-chrom. No.	Total chrom. No.	Translocation $*^{No.}$
BC_1-F_1	**BC_1-F_1-244**	14	13	1	28	0
	BC_1-F_1-245	14	13	1	29	A-d$*^1$
	BC_1-F_1-246	14	13	2	29	0
	BC_1-F_1-247	14	13	1	28	0
	BC_1-F_1-248	15	13	2	30	0
	BC_1-F_1-249	13	13	3	30	A-d$*^1$
	BC_1-F_1-250	14	13	3	31	B.D$*^1$
	BC_1-F_1-251	14	13	1	28	0
	BC_1-F_1-252	14	13	1	28	0
F_2T	**F_2T-254**	14	13	2	29	0
	F_2T-255	14	13	12	39	0

NB: $*^{No.}$ indicates the number of copies.

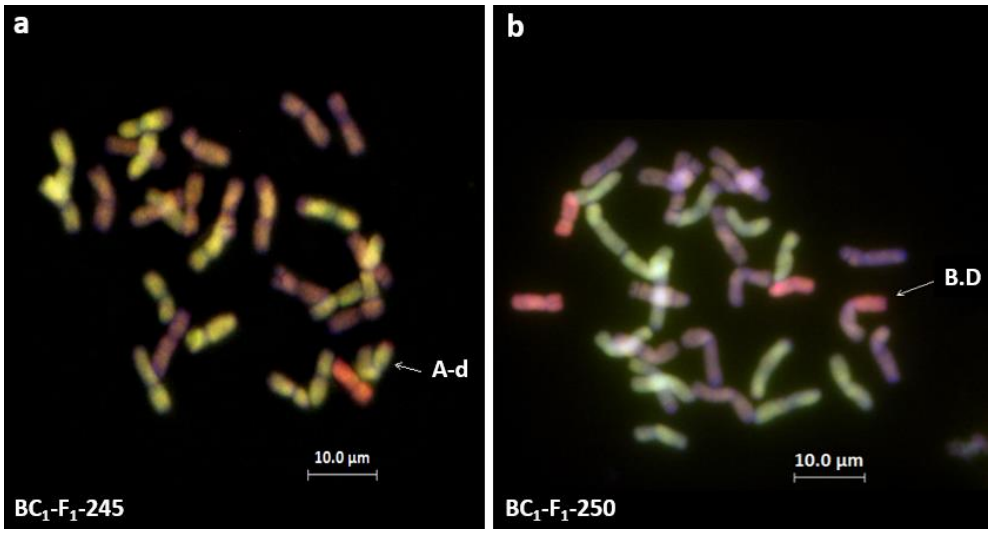

Figure 4-4. Mc-GISH pictures of (a) BC_1-F_1-245 and (b) BC_1-F_1-250 showing the presence of an A-d and B.D translocations, respectively (Chromosome colour code: A-genome: green, B-genome: blue/purple, D-genome: red).

4.3.2.3 Mc-GISH analysis of the BC_1-F_2 and the F_3T lines

A total of 45 lines (36 BC_1-F_2 lines and 9 F_3T lines) were cytogenetically analysed with mc-GISH. BC_1-F_2 lines were assigned the same BC_1-F_1 parental line number code followed by an alphabet letter different between sister lines for easy recognition of the parental lines and identification of the sister lines. Results showed that all the F_3T lines retained at least two D-genome chromosomes, but no translocations could be found (**Table 4-3**).

Table 4-3. Genomic constitution of the F_3T lines revealed by mc-GISH.

No.	F_3T lines reference	A-genome chrom. No.	B-genome chrom. No.	D-genome chrom. No.	Total chrom. No.
1	**F_3T-254-F**	14	12	2	28
2	**F_3T-254-G**	14	12	2	28
3	**F_3T-254-H**	14	13	2	29
4	**F_3T-254-I**	14	14	2	30
5	**F_3T 254-J**	14	14	2	30
6	**F_3T 255-C**	14	14	11	42
7	**F_3T-255-F**	14	12	10	37
8	**F_3T-255-G**	14	13	10	37
9	**F_3T-255-H**	14	14	12	40

NB: Sister lines are grouped together.

Around 65% of the BC_1-F_2 lines (25 lines) had retained at least one D-telomere, chromosome or introgressed D-genome segment. 72% (26 lines) and 20% (2 lines) of the BC_1-F_2 and the F_3T lines, respectively, were tetraploid with a total chromosome number of 28 (**Table 4-3**). Notably, 57.6% (14 lines) of the tetraploid BC_1-F_2 lines had retained at least one of the D-genome chromosomes or introgressed segments (**Table 4-4**) seen in the previous generation. This result suggests that the backcrossing of the F_1T plant to the durum wheat parent helped not only to reduce the chromosome number to restore the tetraploid level, but also increased the chance of chromosome breakage and D-genome introgression, highlighted by the presence of chromosome telomeres and D-genome translocations seen only in the backcrossed lines.

Table 4-4.Genomic constitution of the BC_1-F_2 lines revealed by mc-GISH.

BC_1-F_1 Lines reference	Trans. type*$^{No.}$	BC_1-F_2 lines reference	A-chrom. No.	B-chrom. No.	D-chrom. No.	Trans. type*^{No}	Total chrom. No.
BC_1-F_1-244	-	**BC_1-F_2-244-E**	14	13	0	B.D*^{1}	28
		BC_1-F_2-244-F	14	14	0	-	28
		BC_1-F_2-244-G	14	14	0	-	28
		BC_1-F_2-244-H	14	14	0	-	28
BC_1-F_1-245	A-d*^{1}	**BC_1-F_2-245-G**	14	13	1	-	28
		BC_1-F_2-245-H	13	14	1	A-d*^{1}	29
		BC_1-F_2-245-E	14	14	0	-	28
		BC_1-F_2-245-F	14	14	0	-	28
BC_1-F_1-246	-	**BC_1-F_2-246-E**	14	13+t	1	-	28+
		BC_1-F_2-246-F	14	12	2	-	28
		BC_1-F_2-246-G	13	14	1	A-d*^{1}	29
		BC_1-F_2-246-H	14	13	2	-	29
BC_1-F_1-247	-	**BC_1-F_2-247-E**	14	13	1	-	28
		BC_1-F_2-247-F	14	12	1	B.D*^{1}	28
		BC_1-F_2-247-G	14	13	2	-	29
		BC_1-F_2-247-H	14	14	0	-	28
BC_1-F_1-248	-	**BC_1-F_2-248-F**	14	14	2	-	30
		BC_1-F_2-248-H	15	13	0	B.D*^{1}	29
		BC_1-F_2-248-I	15	13	2	-	29
BC_1-F_1-250	B-D*^{1}	**BC_1-F_2-250-G**	14	14	1+t	A.d*^{1}	29+
		BC_1-F_2-250-H	14	13	0	B.D*^{1}	28
		BC_1-F_2-250-E	14	14	1	-	29
		BC_1-F_2-250-J	14	13	0	B.D*^{1}	28
		BC_1-F_2-250-K	14	13	0	B.D*^{1}	28
		BC_1-F_2-250-L	14	14	2	-	30
BC_1-F_1-251		**BC_1-F_2-251-E**	14	12	2	-	28
		BC_1-F_2-251-G	14	12	2	-	28
		BC_1-F_2-251-H	14	14	t	-	28+t
BC_1-F_1-252	-	**BC_1-F_2-252-E**	14	14	0	-	28
		BC_1-F_2-252-F	14	14	0	-	28
		BC_1-F_2-252-G	14	14	0	-	28
		BC_1-F_2-252-H	14	14	0	-	28
BC_1-F_1-253	-	**BC_1-F_2-253-E**	14	13	1	-	28
		BC_1-F_2-253 F	14	12	2	-	28
		BC_1-F_2-253-G	13	12	3	-	28
		BC_1-F_2-253-H	13	13	2	-	28

NB: *$^{No.}$ indicates the number of copies; +t indicates the presence of telomere; sister lines are grouped together.

Four BC_1-F_2 lines had one A-genome chromosome missing. Two of these lines, however, showed an A-d translocation suggesting that the D-genome segment had been translocated with the missing A-genome chromosome (**Figure 4-5**). Another two BC_1-F_2 sister lines, showed the presence of the extra A-genome chromosome, also seen in the parental line. The number of B-genome chromosomes varied between 12 and 14 (**Table 4-4**), with Mc-FISH analysis revealing 5B to be missing as expected.

No translocations were identified in the F_3T lines (**Table 4-3**), but nine single genomic translocations involving the D-genome with either the A- or the B-genome were identified in the BC_1-F_2 lines (**Table 4-4**), six with the B- and three with the A-genomes. Four of these translocations were identified in the previous generation (**Table 4-4**), indicating that the univalent translocated chromosomes were retained after plant self-fertilisation. Therefore, five new translocations were identified in the BC_1-F_2 lines suggesting that translocations had also occurred in the BC_1-F_2 generation (underlined translocations in **Table 4-4**). Four of the BC_1-F_2 translocation lines identified were tetraploid and thus self-fertilisation of these lines will generate stable homozygous *Ae. tauschii* introgressions into durum wheat.

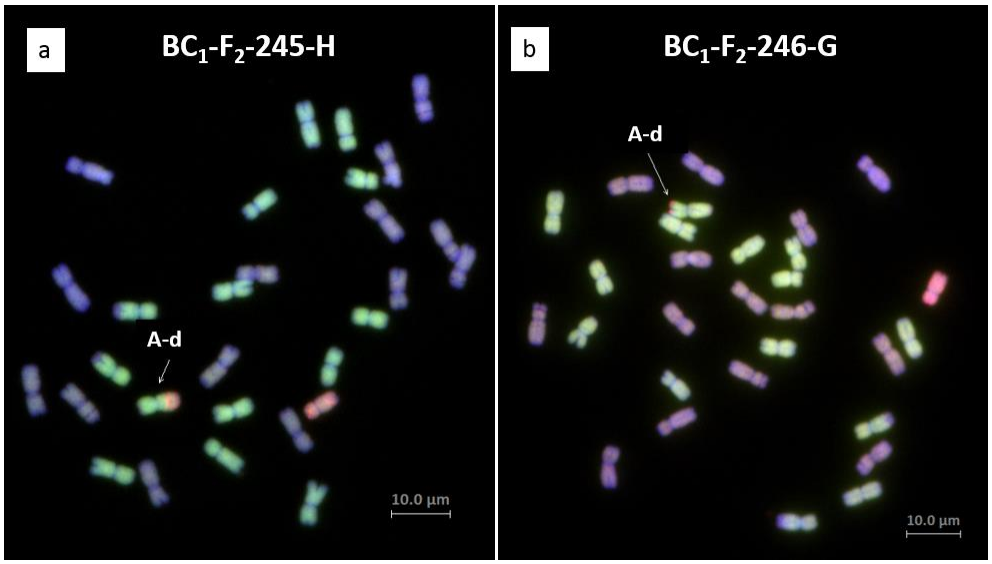

Figure 4-5. Mc-GISH pictures of (a) BC_1-F_2-245-G and (b) BC_1-F_2-246-H, both showing the presence of 12 A-genome chromosomes and an A-D translocation (Chromosome colour code: A-genome: green, B-genome: blue/purple, D-genome: red).

4.3.3 Characterisation of the D-genome retention and introgressions using molecular markers and mc-FISH

4.3.3.1 Genotyping analysis

A total of 31 lines carrying a total chromosome number of 30 or less were genotyped using D-genome specific SSR markers, to identify the linkage group of the retained D-genome chromosome(s) and the introgressed segments. Genotyping results (summarized in **Table 4-5**; green cell highlight the presence of the corresponding locus) of the BC_1-F_2 lines, showed a high retention rate for the 5D chromosomes in most of the lines. A full or a part of 1DS, 1DL, 2DL, 3DL and 4DL chromosome arms were present in some of the lines (**Table 4-5**; Supp. material 8-12).

Table 4-5. Summary of the amplification results of the BC_1-F_2 and the F_3T lines using a set of 21 D-genome specific SSR markers.

Chromosome	1D			2D			3D			4D			5D			6D			7D		
Lines	S	C	L	S	C	L	S	C	L	S	C	L	S	C	L	S	C	L	S	C	L
BC_1-F_2-244-E	0	0	0	0	0	0	0	0	-	0	0	0	0	0	1	0	0	0	0	0	0
BC_1-F_2-245-G	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-245-H	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-246-E	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-246-F	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-246-G	0	0	0	0	0	1	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-246-H	0	0	0	0	1	1	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-247-E	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-247-F	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-247-G	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-248-F	0	0	0	0	0	0	0	0	-	0	1	1	1	1	1	0	0	0	0	0	0
BC_1-F_2-248-H	0	0	0	0	0	0	0	0	-	0	0	0	1	1	0	0	0	0	0	0	0
BC_1-F_2-248-I	0	0	0	0	0	0	0	0	-	0	1	1	1	1	1	0	0	0	0	0	0
BC_1-F_2-250-G	1	0	1	0	0	0	0	0	-	0	0	0	0	0	1	0	0	0	0	0	0
BC_1-F_2-250-H	0	0	0	0	0	0	0	1	-	0	0	0	0	0	0	0	0	0	0	0	0
BC_1-F_2-250-E	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-250-J	0	0	0	0	0	0	0	1	-	0	0	0	0	0	0	0	0	0	0	0	0
BC_1-F_2-250-K	0	0	0	0	0	0	0	1	-	0	0	0	0	0	0	0	0	0	0	0	0
BC_1-F_2-250-L	1	0	1	0	0	0	0	0	-	0	1	1	0	0	1	0	0	0	0	0	0
BC_1-F_2-251-E	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-251-G	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-251-H	0	0	0	0	0	0	0	0	-	0	0	0	1	1	0	0	0	0	0	0	0
BC_1-F_2-253-E	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-253 F	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-253-G	0	0	0	0	1	1	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
BC_1-F_2-253-H	0	0	0	0	1	1	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
F_3T-254-F	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
F_3T-254-G	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
F_3T-254-H	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
F_3T-254-I	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0
F_3T 254-J	0	0	0	0	0	0	0	0	-	0	0	0	1	1	1	0	0	0	0	0	0

NB: S= short arm; c=centromere, L: long arm, 0: no amplification, 1: amplification, -: missing data; sister lines grouped together, green cell highlights the amplification of the corresponding locus.

For example, while the markers for 1DS and 1DL amplified in BC_1-F_2-250-G and -L lines, there was no amplification of the 1Dc marker. This indicate a deletion in the centromeric region confirmed by mc-FISH together with another possible deletion in the telomeric region in the long arm (**Figure 4-6**). The chromosome 5D was the most retained of the D-genome chromosomes (**Table 4-5**) with all of the F_3T lines and 69.2 % (18 lines over 26 lines) of the BC_1-F_2 lines retaining at least one whole 5D-chromosome and another five of the BC_1-F_2 lines retaining either the 5DS or 5DL chromosome arms.

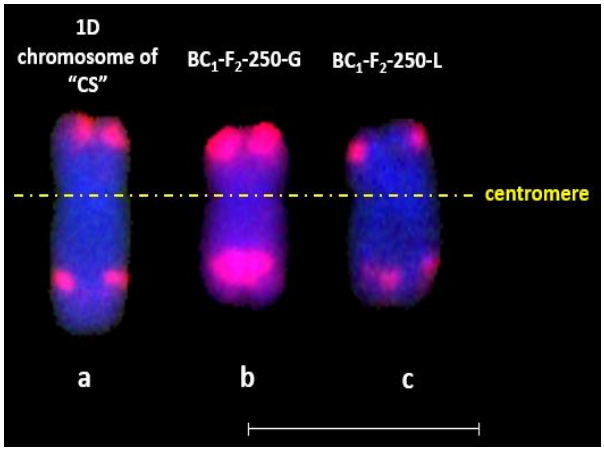

Figure 4-6. Mc-FISH using the Oligo-pAs.1 (red) and Oligo-pSc119.2 (green) probes counterstained with DAPI (blue) of the 1D chromosome of (a) Chinese Spring and 1D chromosomes identified in the two sister lines (b) BC_1-F_2-250-G and (c) BC_1-F_2-250-L. Scale bar, 10µm.

4.3.4 Cytogenetic analysis using mc-FISH

4.3.4.1 The retention of the 5B-chromosome(s) in the BC_1-F_2 and the F_3T lines

The Chinese Spring karyotype was constructed using the Oligo-pAs1-1 and Oligo-pAs1-2 FISH probes labelled in green and red, respectively (**Figure 4-7**, Tang *et al.*, 2014), A FISH based karyotype was then established for all the BC_1-F_2 and the F_3T lines by the allocation of every chromosome to its appropriate group by comparison with the Chinese Spring karyotype. Results confirmed that the B-genome chromosome missing in all the lines was a group 5. The majority of the BC_1-F_2 lines (19 lines = 52.7%) recovered the missing 5B-chromosome. However, six lines (16.6%) had lost the single copy of the 5B-chromosome already present in the BC_1-F_1 parental line. Eleven lines (30.5%) remained heterozygous with a copy of this chromosome still missing (**Figure 4-8**). 44.4% of the F_3T lines had lost the univalent 5B-chromosome copy present

previously in the F_2T plant. In 33.3% of the lines, the second copy of the 5B-chromosome was recovered and in 22.2% of the lines the 5B-chromosome was still univalent (**Figure 4-8**). This result indicates that backcrossing increases the possibility of recovering the missing B-genome chromosome after the use of the substitution lines compared to plant self-fertilisation or a single cross with the durum parent.

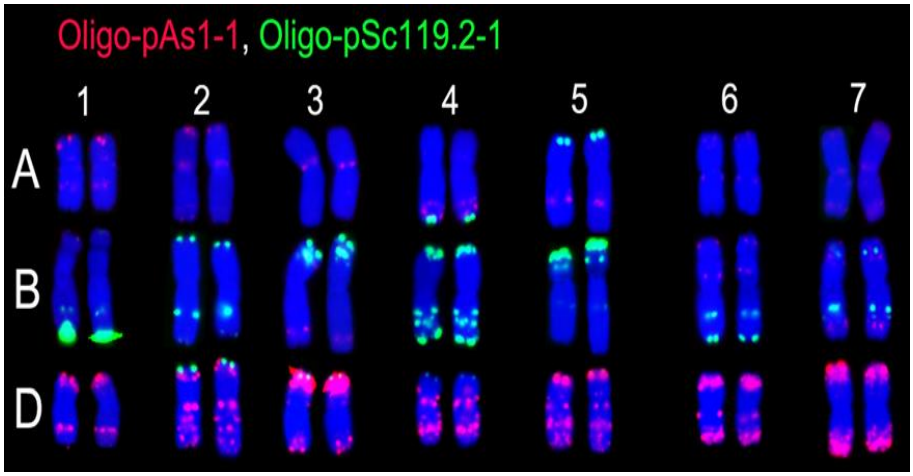

Figure 4-7. Mc-FISH based karyotype analysis using Oligo-pAs1-1 (red), and Oligo-pSc119.2-2 (green) as probes on root tip metaphase chromosomes of Chinese Spring counterstained with DAPI (blue) (Tang *et al.*, 2014).

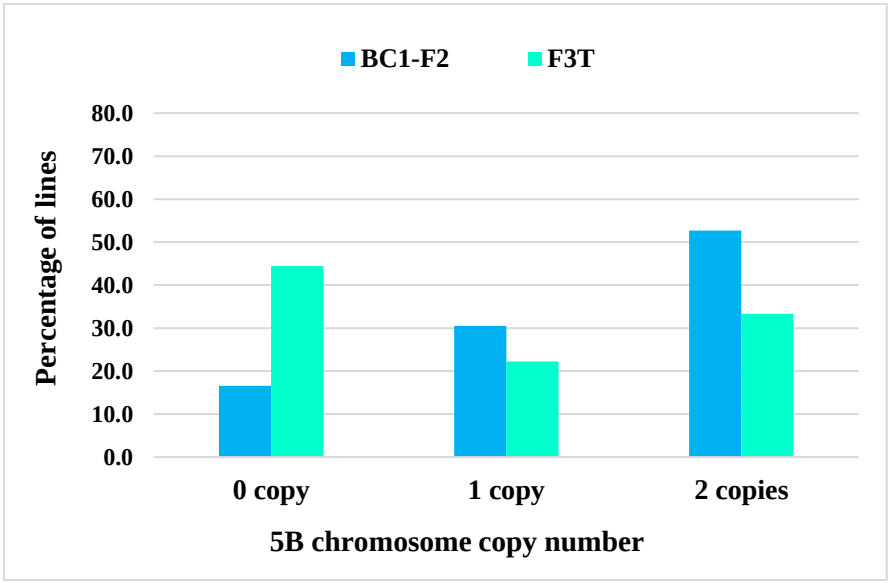

Figure 4-8. The percentage of the BC_1-F_2 and F_3T lines carrying a single copy, a pair or no copy of the 5B chromosome.

4.3.4.2 Mc-FISH characterisation of the introgression lines

FISH based karyotyping of the 31 genotyped lines (**Table 4-5**), confirmed the genotyping results and allowed not only the characterisation of the genomic

composition of all the lines, but also the characterisation of the D-genomic rearrangements and the durum wheat/*Ae. tauschii* introgression lines. In fact, by combining the results of the three methods used, the lines were classified into three different categories:

1-Durum wheat D-genome substitution lines,

2- Durum wheat D-genome addition lines,

3- Durum wheat D-genome introgression lines.

4.3.4.2.1 Charaterisation of the durum wheat D-genome substitution lines

Tetraploid lines with a total of 28 chromosomes, showing the presence of a full D-genome chromosome(s) replacing A- or B-genome chromosome(s) were considered as durum wheat D-genome substitution lines (**Table 4-6**).

Table 4-6. Characterisation of the tetraploid BC_1-F_2 and F_3T D-genome substitution lines.

Pop.	Substitution type	Line ref.	D-chrom. group*No	Rearranged D-chrom. *No.	Substituted chrom. *No.	Total chrom. No.
BC_1-F_2	Monosomic 5D (5B)	**BC_1-F_2-245-G**	5D*1	-	5B*1	28
		BC_1-F_2-246-E	5D*1	-	5B*1	28
		BC_1-F_2-247-E	5D*1	-	5B*1	28
		BC_1-F_2-253-E	5D*1	-	5B*1	28
	Disomic 5D (5B)	**BC_1-F_2-253-F**	5D*2	-	5B*2	28
		BC_1-F_2-251-G	5D*2	-	5B*2	28
		BC_1-F_2-251-E	5D*2	-	5B*2	28
		BC_1-F_2-246-F	5D*2	-	5B*2	28
	Double monosomic	**BC_1-F_2-253-H**	5D*1	5DS.2DL*1	2A*1+ 5B*1	28
	Trisomic	**BC_1-F_2-253-G**	5D*2	5DS.2DL*1	5A*1 + 5B*2	28
F_3T	Disomic 5D (5B)	**F_3T-254-F**	5D*2	-	5B*2	28
		F_3T-254-G	5D*2	-	5B*2	28

NB: *No. indicates the number of copies

Four tetraploid BC_1-F_2 lines; 245-G, 246-E, 247-E and 253-E, showed the presence of a monosomic 5D (5B) substitution, in which one 5B chromosome is substituted by a single copy of a 5D chromosome (e.g. Supp. material 13). FISH based karyotyping results for these lines were consistent with the genotyping results, that showed amplification for all the 5DS, 5Dc and 5DL markers indicating the presence of a full 5D chromosome (**Table 4-5**). In the

BC_1-F_2-246-E line that previously showed the presence of a B-telomere with mc-GISH, mc-FISH analysis confirmed that this telomere is part of the 5BS chromosome arm. Likewise, another four BC_1-F_2 lines, BC_1-F_2-246-F, BC_1-F_2-253-F, and BC_1-F_2-251-E and -G and the two F_3T lines, F_3T-254-F and -G, revealed the presence of a disomic 5D (5B) substitution, with the two 5D chromosomes substituting both copies of 5B, confirming the genotyping results obtained with the 5D-specific SSR markers for these lines.

The analysis of the two tetraploid sister lines; BC_1-F_2-253-G and -H using D-genome specific SSR markers revealed the presence of chromosome arm 2DL as well as chromosome 5D. The FISH karyotype of these two lines showed the presence of a rearranged D-genome chromosome involving a RobT between the 5DS and the 2DL chromosome arms in both of the sister lines. However, the FISH karyotype of BC_1-F_2-253-G also revealed the presence of a pair of 5D-chromosmes, absence of the 5B pair of chromosomes and a single copy of chromosome 5A, forming a disomic 5D (5B) and a monosomic 5DS.2DL (5A) substitution into durum wheat (**Figure 4-9**), whereas in BC_1-F_2-253-H, the rearranged 5DS.2DL chromosome substituted alongside a monosomic 5D chromosome and single copies of the 2A and 5A chromosomes. The presence of the same D-chromosomal rearrangement in both of the sister lines suggests that the translocation occurred in the BC_1-F_1 generation and was retained by both lines after plant self-fertilisation.

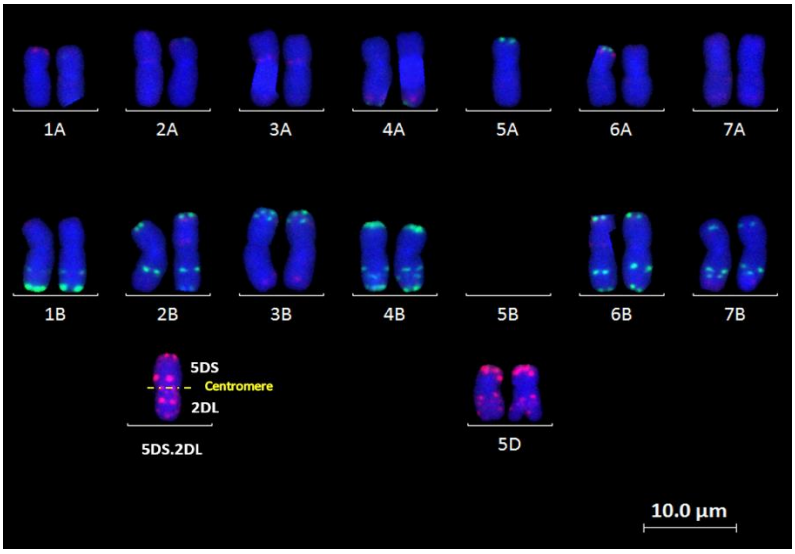

Figure 4-9. FISH-based karyotype of BC_1-F_2-253-G using Oligo-pAs.1 (red) and Oligo-pSc119.2 (green) probes counterstained with DAPI (blue) showing the presence of a 5DS.2DL chromosome translocation.

4.3.4.2.2 Charaterisation of the durum wheat D-genome addition lines

Lines with more than 28 chromosomes, showing the presence of a D-telomere or full D-genome chromosome(s) were considered as durum wheat D-genome addition lines (**Table 4-7**).

Table 4-7.Characterisation of the tetraploid BC_1-F_2 and F_3T D-genome addition lines.

Pop.	Addition type	Line ref.	Chrom. addition*No.	Rearranged D-chrom. *No.	Missing chrom. *No.	Total chrom. No.
BC_1-F_2	Monosomic	**BC_1-F_2-250-E**	5D*1	-	-	29
	Double monosomic	**BC_1-F_2-248-F**	5D*1	5DS.4DL*1	-	30
		BC_1-F_2-250-L	1D*1	4DL-5DL*1	-	30
		BC_1-F_2-246-H	5D*1	5DS.5DL-2DL*1	5B*1	29
	Disomic	**BC_1-F_2-247-G**	5D*2	-	5B*1	29
	Triplosomic	**BC_1-F_2-248-I**	5D*1 + 2A*1	5DS.4DL*1	5B*1	30
	Telocentric	**BC_1-F_2-251-H**	5DS*1	-	-	28+
F_3T	Disomic	**F_3T-254-I**	5D*2	-	-	30
		F_3T-254-H	5D*2	-	5B*1	29
		F_3T 254-J	5D*2	-	5B*1	29

*indicates the number of copies; + indicates the presence of a telomere.

A single D-genome chromosome rearangement was identified in four of the BC_1-F_2 lines. Two sister lines showed the presence of the same rearranged 5DS-4DL chromosome, but these two lines had different genomic compositions (Table 4.7). BC_1-F_2-248-F contained the rearranged chromosome and a univalent 5D-chromosome in a total of 30 chromosomes (a double monosomic tetraploid D-genome addition line) while BC_1-F_2-248-I had extra univalent 2A and 5D chromosomes with a single copy of 5B missing (**Figure 4-10a**). A 4DL-5DL rearranged chromosome was identified in BC_1-F_2-250-L involving the 4DL arm with the telomeric part of the 5DL chromosome arm. The rearrangement was confirmed by the absence of amplification for the 5DS and 5Dc SSR loci and amplification of the 5DL locus. This translocation was visible with mc-FISH which also showed the presence of a 1D univalent chromosome forming a double monosomic durum wheat addition line (**Figure 4-10b**). A translocation of the 2DL chromosome arm into approximately the middle of the long arm of 5D (designated as 5DS.5DL-2DL), was shown to be present in BC_1-

F_2-246-H. A univalent 5D chromosome was also present, but a copy of the 5B chromosome was missing, forming a double monosomic addition line with a total of 29 chromosomes (**Figure 4-10c**).

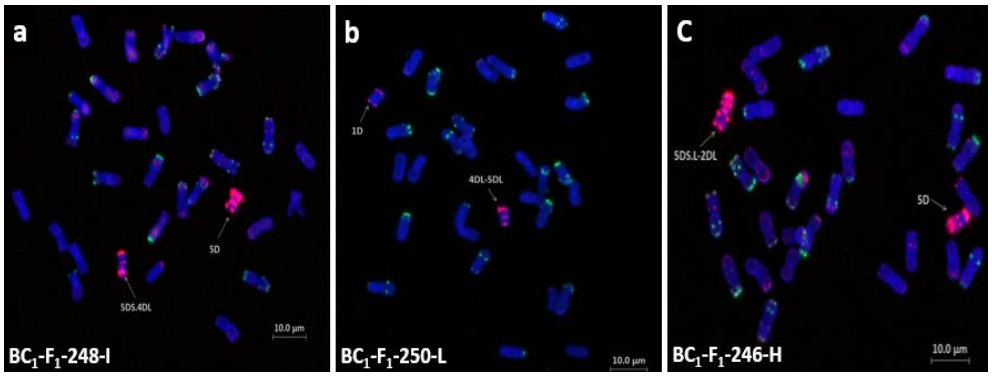

Figure 4-10. FISH-based metaphase spread using Oligo-pAs1-1 (red), and Oligo-pSc119.2-2 (green) as probes counterstained with DAPI (blue) of (a) BC_1-F_2-248-I, (b) BC_1-F_1-250-L and (c) BC_1-F_2-246-H lines showing the presence of rearranged D-genome chromosomes.

A monosomic and disomic 5D addition were identified in BC_1-F_2-250-E and BC_1-F_2-247-G, respectively. BC_1-F_2-250-E showed the presence of a complete set of the A- and the B-genome together with a single 5D chromosome (**Figure 4-11a**). BC_1-F_2-247-G had the same genomic constitution except a 5B-chromosome was missing (**Figure 4-11b**). Finally, BC_1-F_2-251-H had a single 5DS telomere present in addition to the complete set of the A- and the B-genome, as was demonstrated by the amplification of only the 5DS and 5Dc loci in this line (**Figure 4-11c**).

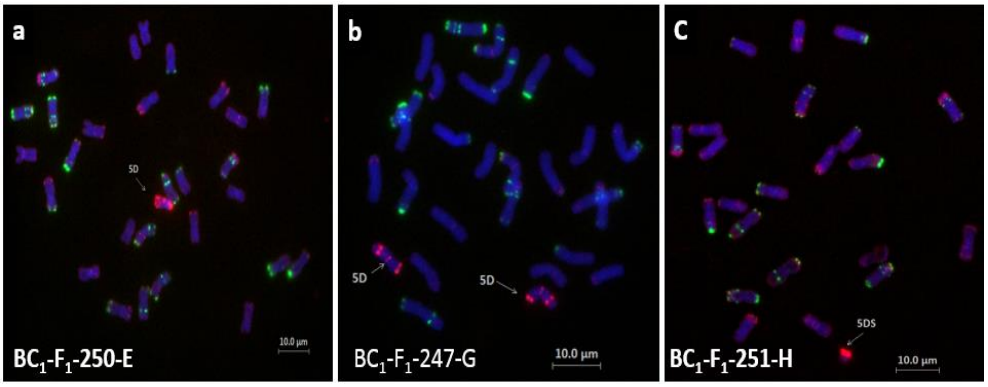

Figure 4-11. FISH-based metaphase spread using Oligo-pAs1-1 (red), and Oligo-pSc119.2-2 (green) as probes counterstained with DAPI (blue) of (a) BC_1-F_1-250-E (b) BC_1-F_2-247-G and (c) BC_1-F_1-251-H lines.

4.3.4.2.3 Charaterisation of the D-genome introgression lines

Mc-FISH analysis of the nine BC_1-F_2 lines showing D-genome introgressions into the A- or the B-genome, allowed the characterisation of these translocations and confirmed the genotyping results. The D-translocations mainly involved the 2DL, 3DL, 5DS and 5DL chromosome arms. These introgression lines were divided into two groups. The first group consisted of tetraploid introgression lines. The second group of lines showed a genomic substitution and/or addition as well as the D-introgression (**Table 4-8**).

Table 4-8.Characterisation the BC_1-F_2 D-genome introgression lines.

Grp.	Lines reference	Lines description	Translocation type*$^{No.}$	Missing chrom. *$^{No.}$	Total chrom. No.
G1	**BC_1-F_2-244-E**	Tetraploid D-genome introgression	5BS.5DL*^{1}	5B*^{1}	28
	BC_1-F_2-250-H		3DL.5BL*^{1}	5B*^{1}	28
	BC_1-F_2-250-J		3DL.5BL*^{1}	5B*^{1}	28
	BC_1-F_2-250-K		3DL.5BL*^{1}	5B*^{1}	28
G2	**BC_1-F_2-247-F**	Monosomic 5D (5B) substitution	5DL.5BL*^{1}	5B*^{2}	28
	BC_1-F_2-248-H	Monosomic 2A addition	5DS.5BL*^{1}	5B*^{1}	29
	BC_1-F_2-245-H	Monosomic 5D addition	5DS-3AS.3AL*^{1}	3A*^{1}	29
	BC_1-F_2-246-G	Monosomic 5D addition	2AS.2AL-2DL*^{1}	2A*^{1}	29
	BC_1-F_2-250-G	Monosomic 1D + 5DL	A-d*^{1}	-	29+t

NB: *$^{No.}$ indicates the number of copies; +t indicates the presence of a telomere.

Tetraploid introgression lines: Four tetraploid lines showed a D-genome introgression into the B-genome. Two different translocations were identified in these lines. The first was a centromeric translocation between the short arm of chromosome 5B and the long arm of chromosome 5D (**Figure 4-12a**) substituting a copy of the 5B chromosome (Supp. material 14). The second translocation identified was a RobT between the long arm of chromosome 3D and the long arm of chromosome 5B in three sister lines (**Figure 4-12b**). Mc-FISH of the three lines revealed that the translocated chromosome substituted a copy of chromosome 5B in a tetraploid background (Supp. material 15). The parental BC_1-F_2-250 line had three D-genome chromosomes in addition to the chromosome carrying the translocation. 50% of the analysed progeny (3 out of

6 lines) retained the translocation and lost all of the three D-genome chromosomes. This can possibly be explained by the pairing between the 5BL translocated arm and the 5BL of its homologous 5B-chromosome during meiosis, facilitating its transmission to the next generation.

D-genomic introgression alongside a D-substitution and/or addition: Five different genomic translocations involving the D-genome with either the A- or the B-genome were identified in this second group (**Table 4-8**). Mc-FISH of the BC_1-F_2-247-F line revealed the presence of a RobT between the long arm of chromosome 5D and the long arm of chromosome 5B in a tetraploid background (**Figure 4-12c**). The FISH based karyotype of this line showed that the translocated chromosome substituted a 5B chromosome alongside a 5D (5B) substitution of the second copy of chromosome 5B (e.g. **Figure 4-13**). The D-specific SSR marker confirmed the presence of the 5D chromosome in this line. A RobT between the short arm of chromosome 5D and the long arm of chromosome 5B was identified in BC_1-F_2-248-H, substituting a copy of chromosome 5B (**Figure 4-12d**). The presence of the 5DS chromosome arm was confirmed by the 5Ds and the 5Dc markers. An extra copy of chromosome 2A was distinguished by mc-FISH in a total of 29 chromosome. The investigation by mc-GISH of the telomeric A-d translocation identified in BC_1-F_2-245-H characterised it as a small D-genome segment in the short arm of an A-genome chromosome. Mc-FISH analysis revealed that this translocation most probably happened with chromosome 3A (**Figure 4-12e**). A full 5D chromosome was identified as an addition in this line. The amplification for the 5D chromosome markers in these lines confirmed not only the monosomic 5D addition but also indicated that the translocated small D-genome segment may belong to chromosome 5B. When compared to the wheat-FISH karyotype, the strong red dots visualised by mc-FISH may indicate that the small D-genome segment introgressed probably belongs to the telomeric region of the 5DS chromosome arm. BC_1-F_2-246-G showed the presence of a new A-d translocation. Based on the mc-FISH karyotype, the D-genome segment introgressed was identified as part of the long arm of chromosome 2D translocated with chromosome 2A in the middle of its long arm (**Figure 4-12f**). The presence of the 2DS segment in this line was confirmed by the amplification

of the 2DS SSR locus. Karyotype analysis showed that the translocated chromosome replaced a copy of chromosome 2A with a univalent copy of chromosome 5D retained as a monosomic addition.

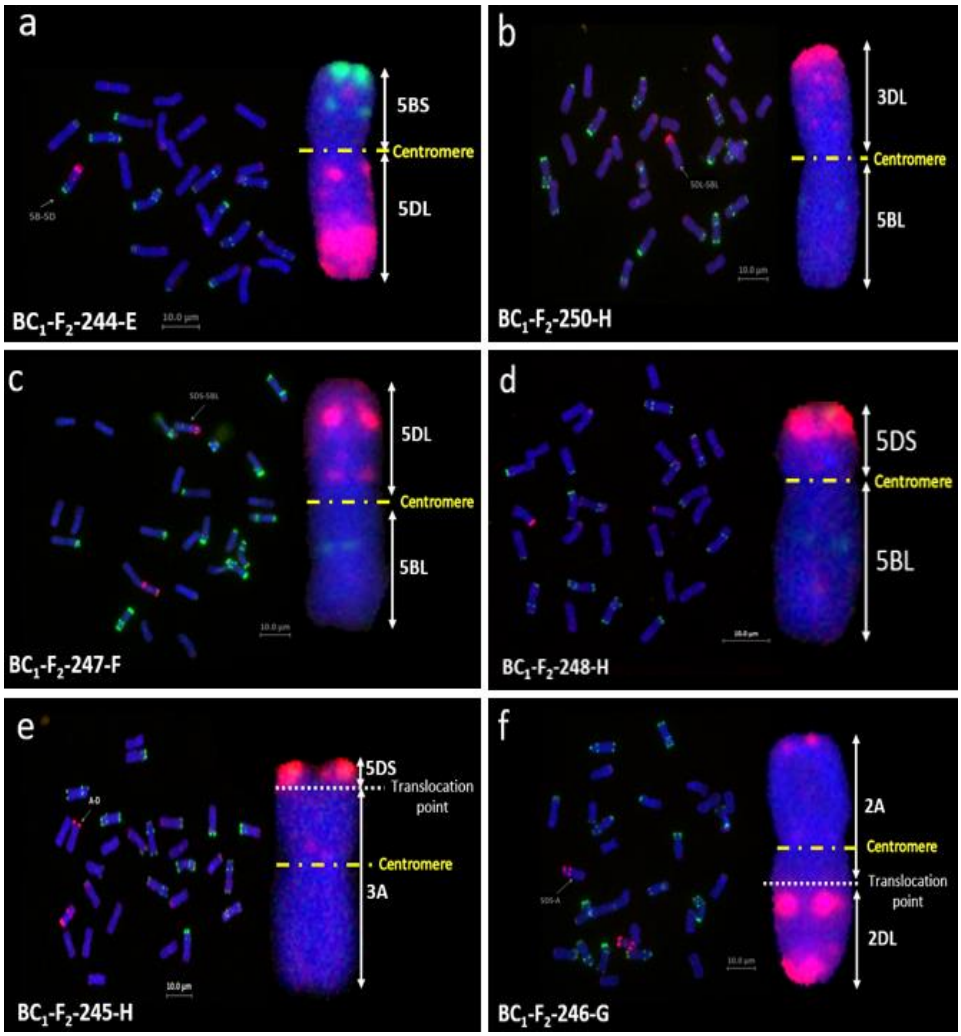

Figure 4-12. Genomic composition of BC_1-F_2 lines using mc-FISH (b) probed with Oligo-pAs.1 (red) and Oligo-pSc119.2 (green) counterstained with DAPI (blue) showing the presence of a genomic translocation involving the D-genome.

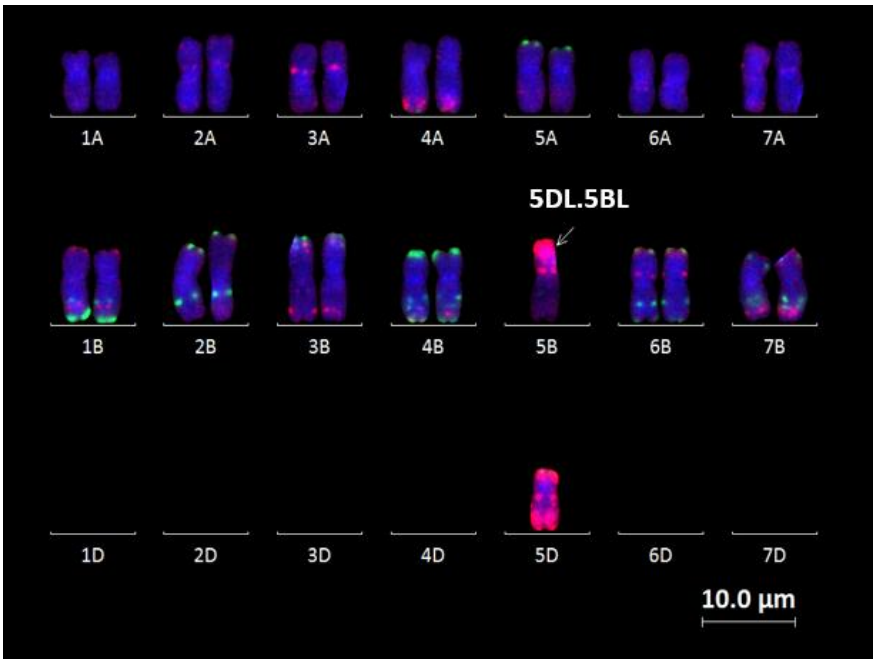

Figure 4-13. FISH based karyotype of the metaphase spread of BC_1-F_2-247-F using Oligo-pAs.1 (red) and Oligo-pSc119.2 (green) probes counterstained with DAPI (blue).

Another telomeric A-d type translocation found in BC_1-F_2-250-G, was visible only with mc-GISH and couldn't be seen by mc-FISH, presumably because of the very small size of the introgressed D-genome segment. Thus, it wasn't clear with which A-genome chromosome the translocation occurred. Mc-FISH based karyotyping of this line, supported by the molecular results, also showed the retention of chromosome 1D and a 5DL telomere as an addition in a total of 29 chromosomes plus a telomere.

4.3.5 Screening for the introgression of Puroindoline genes in durum wheat

The presence of the Puroindoline genes; *Puroindoline a* (*Pina*) and *Puroindoline b* (*Pinb*) located at the *Hardness* (*Ha*) locus (Bhave and Morris, 2008a) on the 5DS chromosome arm, in BC_1-F_2-248-H showing a 5DS introgression was investigated using the two corresponding STS markers. The durum wheat parent "Om Rabiaa 5" was used as a negative control together with the Chinese Spring, LND 5D (5B) and *Ae. tauschii* P99 as positive controls. The amplification of both markers in BC_1-F_2-248-H confirmed the introgression of both genes responsible for the soft kernel texture in wheat in this tetraploid line (**Figure 4-14**).

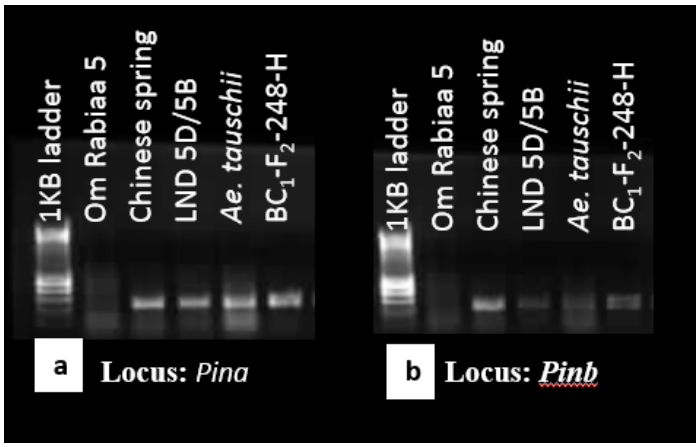

Figure 4-14. Agarose-gel using the (a) *Pina* and (b) *Pinb* STS markers shown the presence of both Puroindoline genes in the BC_1-F_2-250-L introgression line.

4.4 Discussion

4.4.1 Seed production and population development

A very low crossability level between *Ae. tauschii* and the tetraploid substitution lines was seen in this work with only four F_1 hybrid seeds produced out of the 20 crosses made. A low crossability level was also described by Zhang *et al.* (2008) when crossing LND 5D (5B) substitution line to *Ae. tauschii*, with a crossability percentage of 0.56%. In addition, testing the crossability level between *Ae. tauschii* and all the Langdon D-genome disomic substitution lines, showed that the normal "Langdon" cv. carries dominant alleles on the 7A and the 4B-chromosomes responsible for inhibiting the crossability with *Ae. tauschii* (Zhang *et al.*, 2008).

The F_1 hybrid plant had tough tenacious glumes, a trait obviously inherited from *Ae. tauschii* and also showed a male sterile phenotype. As expected, cytological observation via mc-GISH of the root-tip cells confirmed that the F_1 hybrid plant was triploid with 21 chromosomes, indicating that it was a true hybrid. Chromosomes were determined to be seven univalent A-genome chromosomes, six univalent B-genome chromosome and eight univalent D-genome chromosomes with the absence of the 5B-chromosome and the presence of a pair of the 5D-chromosomes, resulting from the use of the LND 5B(5D) substitution line. Out of the four F_1 hybrid seeds obtained, only one germinated normally and reached the adult stage to produce only one F_1T seed. This low fertility level of the F_1 hybrid could be explained by its unequal chromosome number causing problems of pairing at meiosis. The inspection of the metaphase spread of the F_1T plant showed that a copy of the A- and the B-

genome chromosomes were recovered, and eight D-genome chromosomes were retained after crossing to the durum parent. Two F_2T and nine BC_1-F_1 lines were successfully germinated to produce the F_3T and BC_1-F_2 generations. These lines showed an increased fertility level compared to the previous generation, reflected by the seed set. This gradual improvement in fertility was due to the gradual restoration of the tetraploid number of chromosomes (28) and the reduction in the retention of univalent D-genome chromosomes after backcrossing and selfing.

4.4.2 Screening and characterisation of genomic translocations involving the D-genome

The absence of the *Ph1* locus in the F_1 gamete promoted the occurrence of a single A-d translocation which was detected in the F_1T plant. Newly formed translocations involving the D-genome were identified in one BC_1-F_1 line as well as five BC_1-F_2 lines, indicating that translocation can still occur in the advanced self-fertilisation of the backcross generations, in the presence of the *Ph1* locus. Notably, most of the translocations identified were of the RobT type, with more of the D-genome joined to the B-genome than joined to the A-genome. RobT are produced by the centromeric breakage-fusion of univalents in double monosomic plants (Sears, 1952; reviewed in Zhang *et al.*, 2015). In fact, only 13 B-genome chromosomes were counted in the F_1T plant and the BC_1-F_1 lines indicating the presence of a univalent copy of the 5B-chromosome. The univalent state of the 5B chromosome and the D-genome chromosomes in these lines promoted the production of four different B.D RobT. As was seen using mc-FISH and D-genome specific SSR markers, these RobT involved either arm of chromosome 5B with one of the 3DL, 5DS or 5DL chromosome arms, generating the 5BS.5DL, 3DL.5BL, 5DL.5BL and, 5DS.5BL translocations. Centric breakage of a univalent in wheat may take place in the first or second meiotic anaphase, with several different types of breakage in each (Sears, 1952). In the first division, the breakage may involve separation of the arms (breakage across both sister chromatids) or separation of a single chromatid of one arm from the remainder of the chromosome. Separation of sister chromatids in the first division may lead to breakage across the centromere in the second division, producing telocentric chromosomes. Hence,

rearrangements caused by chromosome breakage followed by fusion of the broken ends can be expected. This centromeric breakage of univalent chromosomes was used to generate and to manipulate translocations of alien chromosome arms into wheat (Lukaszewski, 1993, 1997). The most common alien introgression in wheat, translocation 1RS.1BL, is a result of the centric division and fusion of the broken arms of the chromosome 1R of Rye and 1B of wheat (Mettin *et al.,* 1973; Schlegel and Korzun, 1997).

As mentioned above a univalent 5B copy was present in the parental line of all the lines with RobTs indicating the presence of a single dose of the *Ph1* gene. With this in mind, using monosomic lines of common wheat, Vega and Feldman, (1998) studied the effect of the *Ph1* gene on centromere division of univalents at meiosis. Results showed that centric breakage of univalents was higher in the presence of the normal two doses of *Ph1*, compared to the complete absence of this gene, suggesting that *Ph1* is a *trans*-acting gene affecting centromere-microtubule interaction (Vega and Feldman, 1998). Knowing that the *Ph1* gene was proven to be a hemizygous-effective gene in chromosome pairing (e.g. its single dose has an equal effect as its double dose) (Jauhar *et al.,* 1991; 1999), our results indicated that this gene is also hemizygous-effective on centromeric division of univalent chromosomes in meiosis.

Mc-FISH helped to confirm the results obtained by SSR marker analysis and also to characterise the translocated chromosomes and identify the genomic composition of each line. This efficiency of combining mc-GISH with mc-FISH on wheat alien hybrids to enable chromosomes belonging to different genomes to be detected and identified has been shown previously, such that intergenomic rearrangements within a polyploid species can be visualized (Nagy *et al.,* 2002; He *et al.,* 2017).

4.4.3 Tetraploid D-genome introgression lines

Five tetraploid lines showing *Ae. tauschii* introgressions in the BC_1-F_2 generation were identified, with three of them carrying the same 3DL.5BL RobT, retained from their parental line and substituting a copy of the 5B chromosome in a total of 28 chromosomes. Homozygous 3DL *Ae. tauschii*/ durum wheat introgressions can be used to study yield related traits in durum wheat such as spike number or grain weight. Indeed, Pestsova *et al.* (2006)

distinguished the presence of two favourable QTLs for grain weight per ear and spike number on chromosome arm 3DL of *Ae. tauschii* mapped in *T. aestivum-Ae. tauschii* introgression lines.

The RobT 5BS.5DL, identified in the tetraploid BC_1-F_2-244-E line, also substituted a 5B chromosome. A tetraploid homozygous line for the 5BS.5DL translocation will act like *ph1* mutant because of the missing 5BL chromosome arms. Equally important, the translocated 5BS arm, is known to carry a gene that promotes pairing in its distal third (Riley and Chapman, 1967; Feldman and Mello-Sampayo, 1967; Kota and Dvořák, 1986). In addition, the chromosome arm 5DL also known to carry a pairing promoter genes (Feldman, 1966). The presence of this gene was confirmed after the decrease in chiasma frequency in nulli-5D plants by Sears (1976). Taking this into consideration, a tetraploid homozygous 5BS-5DL line can potentially promote more homoeologous pairing in wheat when compared to the disomic 5D (5B) substitution line. Therefore, the offspring of the homozygous 5BS.5DL tetraploid lines can be checked with mc-GISH for the presence of genomic rearrangement. *T. aestivum*, either homozygous for the *ph1b* allele or nullisomic for chromosome 5B, showed the presence of chromosomal rearrangements (Sanchez-Moran *et al.*, 2001). The frequency of the occurrence of genomic translocations using this particular line can also be assessed in the progeny of a crossing program to several alien species. Another tetraploid BC_1-F_2 line showing a 5BL.5DL translocation was identified in BC_1-F_2-247-F. This translocation substituted a 5B-chromosome together with another monosomic 5D (5B) substitution. Progeny of this line are expected to segregate for the retention of the 5D and/or the translocated chromosome.

4.4.4 D-genome introgression lines in the presence of monosomic additions

Four BC_1-F_2 lines were identified with single genomic translocations involving the D-genome but shown to have a genomic composition of 29 chromosomes due to the presence of monosomic 5D, 1D or 2A additions. For example, three copies of chromosome 2A were present in the BC_1-F_2-248-H line derived from a BC_1-F_1 lines with fifteen A-genome chromosomes which suggested the occurrence of an abnormal mitosis originally in the F_1T line. Two

further BC_1-F_2 lines had a copy of the 5A-chromosome missing, with a copy of chromosome 2A missing as well in one. These results indicate that it is possible that the stress caused by the unequal genomic composition in these lines and/or the presence of monosomic chromosomes of *Ae. tauschii* might be one of the factors that induced abnormal mitosis, resulting in the elimination or the retention of extra copies of some A-genome chromosomes. In fact, our results are consistent with previous studies which indicated that abnormal mitotic behaviour in wheat could be induced by either allopolyploidization or monosomic additions of alien chromosomes. Abnormal mitotic behaviour was observed in the progeny of *T. aestivum* × *Thinopyrum ponticum* amphiploids (Brasileiro-Vidal *et al.,* 2005). In addition, selfed progeny of wheat-rye monosomic addition lines such as 1R, 4R and 6R showed an abnormal meiotic behaviour of wheat chromosomes through the elimination or the addition of some of the wheat chromosomes (Fu *et al.,* 2012; 2013), e.g., three 4A-chromosomes were observed in one of the progeny from a 7R monosomic addition line, whereas, 5A and 4B chromosomes were eliminated from some of the progeny of the 6R monosomic addition line.

One 5DS introgression consisted of the very distal part of the 5DS arm into the short arm of chromosome 3A. This translocation occurred in the F_1T line and was caused by the absence of the *Ph1* gene. As mentioned above in section 4.4.2, new translocations were identified in the BC_1-F_2 generation, of which two single A-d type were found in two BC_1-F_2 lines. The small D-genome segment introgressed into the distal region of an A-genome chromosome short arm, could be phenotyped for traits of interest. If the line is shown to carry a favourable trait such as disease resistance, the translocated segment can be further characterised using molecular markers such as the 90K SNP array. The second new A-d translocation consisted of a non-centromeric translocation of the distal part of the 2DL chromosome arm into approximately the middle of the long arm of the chromosome 2A.

Lines with homozygous translocations involving the D-genome of *Ae. tauschii* but without the monosomic additions will be selected in the next selfing generation(s). Monosomic additions in wheat showed a low transmission rate after plant selfing (Makino, 1981; Dhaliwal *et al.,* 1990). As described previously for univalent chromosomes, monosomic addition chromosomes tend

to undergo centromeric breakage producing telocentrics that are mainly lost through meiosis. Once the lines are homozygous for the translocations (and hence the translocation is stable) it will be possible to multiply the lines and screen them for different traits. For example, Pestsova *et al*. (2006) detected the presence of a favourable QTL with additive effect on fertility and grain weight on *Ae. tauschii* 2DL by using *T. aestivum-Ae. tauschii* introgression lines.

4.4.5 Introgression of Puroindoline genes in durum wheat

Durum wheat is a leading cereal grain whose primary use is the production of semolina and pasta characterised by a very hard kernel texture. The distal part of 5DS contains two *puroindoline* genes, *Puroindoline a* (*Pina-D1*) and *Puroindoline b* (*Pinb-D1*) coded at the *Hardness* locus and responsible for the soft kernel trait in wheat (reviewed in Morris, 2002; Bhave and Morris, 2008a, b). BC_1-F_2-248-H, which showed a 5DS chromosome arm introgression, was assessed for the presence of these two genes. Molecular marker analysis confirmed the presence of these genes. By the same means, using the LND 5D (5B) substitution line as a source of the 5D-chromosome, Morris *et al.* (2011) were able to develop soft kernel durum wheat through the generation of a 5DS-5BS.5BL translocation with a *ph1b*- mediated homoeologous recombination from the 5D of Chinese Spring. The 5D segment was characterised as very small in size and determined to be ~24.36-Mbp, whereas a ~20.01-Mbp segment of chromosome 5BS was lost (Jeffrey *et al.,* 2017). Furthermore, characterization of *Ae. tauschii* and synthetic hexaploid wheat has identified eight different *Pina* alleles and six unique *Pinb* alleles that are all associated with a soft endosperm (Gedye *et al.,* 2004; Massa *et al.,* 2004). Some of these may confer a softer kernel texture than that endemic to *T. aestivum* (Gedye *et al.,* 2004). Presuming that recombination may have occurred between the 5DS arm of *Ae. tauschii* and the 5DS arm of wheat in BC_1-F_2-248H, this line might be of interest to study the potential allelic variation in the *puroindoline* genes introgressed from *Ae. tauschii* into durum wheat.

4.4.6 The characterisation of the D-rearranged chromosomes

All of the D-rearranged chromosomes involved either the short or the long arm of chromosome 5D with the long arm of chromosomes 2DL or 4DL, suggesting that these chromosomes are the most vulnerable for breakage in a

univalent state. Centromere breakage and fusion were shown to occur in the F_1 of wheat-rye hybrids (Lukaszewski and Gustafson 1983; Friebe and Larter, 1988). However, in the F_1 hybrids produced here, 5D was present as a chromosome pair. For this reason, the D- rearrangements are most likely to have occurred in the F_1T gametes where all D-genome chromosomes, including the 5D were univalent, making them prone to mis-division followed by fusion leading to rearrangement.

The 5DS.4DL RobT was found as an addition alongside a 5D-. A copy of the 5B-chromosome was still missing in one of the lines carrying this RobT, suggesting that a potential substitution line involving this rearranged chromosome could be selected in the next selfed generation. A previous study showed the impact of a 4DL introgression carrying the *Kna1* locus from bread wheat which enhanced the salt tolerance of the durum wheat (Dvořák *et al.*, 1994). The introgression of *Ae. tauschii* 4DL into durum wheat could again potentially lead to the study of the allelic variation at this locus compared to the bread wheat allele. The presence of the rearranged D-genome chromosomes in sister lines demonstrated that this univalent rearrangement can be stable and successfully retained in selfed-generations.

A deletion was identified in a 1D chromosome via SSR markers by the absence of the amplification for the 1Dc locus and confirmed by mc-FISH. The presence of deletions indicates the occurrence of Breakage-Fusion-Bridge (BFB) cycles. BFB cycles begin when a telomere breaks off a chromosome. When the damaged chromosome replicates, its sister chromatids fuse and form a bridge during anaphase, with the two centromeres of the fused sister chromatids pulled to opposite poles of the dividing cell. After the bridge breaks, the resulting daughter cells receive defective chromosomes that lack telomeres and can initiate new BFB cycles (Lukaszewski, 1995). The BFB cycle is recognized as one of the mechanisms that causes genome instability producing aberrations, inversions and inter-chromosomal translocations as well as the deletion seen in this work (Gisselsson *et al.*, 2000; MacKinnon and Campbell, 2011).

More examples of alien introgression through a spontaneous translocation with the 5D chromosome are found in the literature. For instance, Kruppa *et al.* (2013) reported the development of a 4HL.5DL RobT line after crossing the 4H

(4D) wheat–barley substitution line with the Chinese Spring *ph1b* mutant. The introgression of the 5M^{g}#1 chromosome from *Ae. geniculata* into wheat, took place through two spontaneous translocations with chromosome 5D resulting in a distal 5DL-5M^{g}#1L.5M^{g}#1S translocation and an interstitial recombinant chromosome into the 5D chromosome designated as 5DS.5DL-5M^{g}#1L-5DL (Liu *et al.,* 2011). The introgression of the 1R segment of rye through a translocation in the long arm of 5D was induced by the gametocidal system (Masoudi-Nejad *et al.,* 2002). More recently, *Agropyron elongatum* chromosome arms belonging to homoeologous group 5 were introgressed into wheat through a translocation with 5DL (Li *et al.,* 2017). As the case with our results, these studies suggest that the 5D chromosome is the most vulnerable to chromosomal breakage in wheat/alien species hybrids, making it more receptive for introgressions. Study of the transmission rate of different rearranged D-genome chromosomes when present in tetraploid wheat lines, either as double monosomic substitution or addition lines would be very interesting and would help to establish their potential for introgression.

Conclusion

The hypothesis of using the Langdon 5D (5B) disomic substitution for the production of durum wheat/*Ae. tauschii* introgression line was seen to be a successful strategy. Introgression lines were developed and future selfing of the line will allow the selection of a stable homozygous introgression lines. Next steps will include the development and the use of D-genome specific KASP markers that will help characterise the introgression lines.

5 Chapter V. The introgression of D-genomic rearrangements identified in wheat/*Am. muticum* introgression lines into durum wheat

5.1 Introduction

Wheat production is dependent on the performance of high yielding varieties that are enriched with alleles to sustain resistance/tolerance to biotic and abiotic stresses. Thus, the incorporation of new alleles into elite wheat germplasm has long been recognized as an essential component of improving wheat productivity and securing global wheat supply. Bread wheat could be an excellent source of genetic enhancement of durum wheat, due to the shared AABB tetraploid chromosomes and the potential for the transfer of desirable D-genome loci into durum. Although genetic variability can be exchanged between *T. durum* and *T. aestivum*, there is little, if any chance, for gene exchange from the D-genome in the presence of the *Ph1* gene (Riley and Chapman, 1958). To overcome the effect of *Ph1*, several strategies can be used to introgress the D-genome into durum such as transformation or the use of *ph1* mutant genotypes.

Wheat related species can be used as important sources of genetic variation. Several studies have focussed on gene introgression into bread wheat from its wild relative species (reviewed in Molnár-Láng *et al.*, 2015). As described previously in chapter 1, *ph1* mutant wheat genotypes are mainly used to promote paring between homoeologous chromosomes and hence chromosomal exchange between the alien and the wheat chromosomes. Therefore, pairing between the A, B and D sub-genomes of wheat is also possible leading to wheat sub-genomic rearrangement.

Contrary to the translocation of the alien segment that can be easily identified via molecular marker, it is difficult or almost impossible to identify wheat sub-genomic rearrangements with molecular markers when they are a single translocation in the presence of the homologous copy of the translocated segment. Moreover, as was used in the past, cytogenetic analysis via single colour FISH or GISH enable only the detection of the sequence of interest or the alien chromatin, respectively (e.g. Heslop-Harrison *et al.*, 1990; Yamamoto and Mukai 1990; Leitch *et al.*, 1991). With the advances in the field of

cytogenetics, it became possible to simultaneously identify the presence of wheat sub-genomic rearrangements and the alien introgression. In fact, the development of a variety of probe labelling procedures, allowed the simultaneous detection of different genomes through the use of multicolour GISH and FISH (Reid *et al.,* 1992; Mukai *et al.,* 1993). Utilizing genomic DNA as a labelled probe, GISH can identify parental chromosomes by genome, highlight translocations, and detect chromosomal rearrangements. This approach proved to be a very effective way to confirm the hybrid status after crossing and the identification of genomic rearrangements and introgressions in wheat (Nemeth *et al.,* 2015; King *et al.,* 2017).

In wheat breeding programs using wide hybridization methods to improve wheat cultivars, attention was often directed at the alien chromatin introgression. However, wheat genomic alteration and translocation in the derivative wheat/ alien introgression lines are either unnoticeable or discarded as they are not of interest. Hypothetically, breeders select against the lines carrying wheat sub-genomic rearrangements and select for the unique presence of alien introgressions in a clear genomic background of wheat. Lines showing an alien introgression of a particular genomic region of interest, alongside wheat sub-genomic rearrangements, can be backcrossed to wheat with the aim to discard these rearrangements and retain the alien introgression.

5.1.1 D-genome of bread wheat introgressions into durum wheat

The use of the D-genome of bread wheat for durum wheat improvement has an advantage over synthetic wheats that contain a full copy of the D-genome of the wild species *Ae. tauschii*, since the D-genome of bread wheat is already fixed for major domestication traits. According to reports in the literature, only a few genomic regions harbouring a gene of interest on the D-genome of bread wheat, have been directly targeted and introgressed into durum. These genes were related to either abiotic stress tolerance, biotic stress resistance or end-use quality (Han *et al.,* 2014, Xu *et al.,* 2005). For instance, the introgression of the 4D chromosome segment via recombination with the 4B chromosome in the genetic background of durum wheat increased its salt tolerance via the introgression of the *Kna1* locus on the 4DL chromosome arm (Dvořák *et al.,* 1994). Similarly, a 4D chromosome fragment introgression from bread wheat,

provides aluminium tolerance in an elite durum wheat cultivar (Han *et al.,* 2014).

Examples of gene introgression for biotic stress include the transfer of the *Lr34/Yr18/Sr57/Pm38/Ltn1* resistance genes, located on the D-genome of bread wheat providing a broad-spectrum resistance to wheat leaf rust (*Lr34*), yellow rust (*Yr18*), stem rust (*Sr57*) and powdery mildew (*Pm38*) pathogens. The partial resistance provided by these genes is only apparent in the adult stage and is not effective in field-grown seedlings. Using transformation, these genes have been transferred to the durum wheat cultivar "Stewart" (Rinaldo *et al.,* 2017). Transgenic plants showed robust seedling resistance to pathogens causing wheat leaf rust, yellow rust and powdery mildew disease. These results demonstrate that expression of a highly durable, broad-spectrum adult plant resistance gene in bread wheat can be introgressed to provide seedling resistance in durum wheat (Rinaldo *et al.,* 2017).

With the increasing interest in the development of durum wheat cultivars with a dual-purpose end-use, a possible strategy to realise further improvement in bread-making characteristics of durum wheat is the introgression of the glutenin subunits associated with the D-genome in bread wheat (Ciaffi *et al*., 1995; Gennaro *et al*., 2012). These glutenin subunits, encoded by the *Glu-D1d* allele, are located on chromosome arm 1DL. Joppa *et al.* (1998) developed a number of durum 1A-1D translocation lines carrying *Glu-D1d*, by spontaneous translocations, using the Langdon 1D (1A) disomic substitution lines. Other studies used *ph1* mutant-induced 1A-1D or 1B-1D translocations to introgress this allele into durum wheat (Ceoloni *et al*., 1996, Vitellozzi *et al*., 1997, Blanco *et al.*, 2002, Lukaszewski, 2003).

5.1.2 The generation of alien introgression in wheat

Wheat is related to a large number of other species, many of which are wild and uncultivated. The wild relatives, unlike wheat, provide a vast and untapped reservoir of genetic variation for potentially most, if not all, agronomically important traits (Friebe *et al.,* 1996; Jauhar and Chibbar, 1999; Qi *et al.,* 2007; Schneider *et al.,* 2008). Attempts have been made to exploit the genetic variation from the wild species. Transfer of alien chromatin starts with an interspecific cross between wheat and the target alien species, which results in

the generation of amphiploids. Amphiploids are hybrids that contain a haploid set of chromosomes from both parents; in this case this includes wheat and an alien species. Next, backcrossing is required to generate addition, substitution, translocation, and/or recombinant lines, the latter two events occurring either spontaneously, through DNA breaks, or via perturbation of the regulators of chromosome pairing. In normal circumstances, chromosome pairing occurs between homologous chromosomes and is tightly regulated by the *Ph1* gene (Al-Kaff *et al.,* 2008; Griffiths *et al.*, 2006; Riley and Chapman, 1958; Sears and Okamoto, 1958; Mello-Sampayo, 1971). Thus, the *Ph1* locus normally has to be removed before homoeologous recombination between the chromosomes of a wild relative and wheat can occur (Al-Kaff *et al.,* 2008; Sears, 1977). However, some alien species carry gene(s) which supresses the *Ph1* locus, thus enabling recombination to occur directly between homoeologous chromosomes in the interspecific hybrid. Inhibition by alien species genes of the wheat *Ph1* system has mostly been characterized in *Ae. speltoides* (Li *et al.,* 2017). However, many studies demonstrate that genes affecting the wheat diploidizing control system exist in several other species of *Aegilops*, *Secale*, *Agropyron* or *Elymus*. The presence of homoeologous pairing promoting genes in the diploids *Ae. longissima* and *Am. muticum* were confirmed as early as that reported for *Ae. speltoides* (Riley, 1966; Mello-Sampayo, 1971; Dover and Riley, 1972). The size of an introgression can be highly variable. Size is dependent on compatible regions for recombination, as many alien chromosomes are rearranged relative to the homologous chromosomes of wheat (Devos *et al.,* 1993). Often the sites of wheat–alien recombination are unevenly distributed over the chromosome such that telomeric regions recombine more readily than pericentromeric regions (Curtis and Lukaszewski, 1991).

5.1.3 Genomic rearrangements and chromosomal structural variations in the derivative lines of wheat/alien hybrids

Several studies highlighted the presence of chromosomal alteration and translocation of the wheat genome in the derivative alien/wheat introgression lines. Chromosome rearrangements, including deletions, translocations, dicentric chromosomes and a paracentric inversion, were observed during the production of the $6B^s$ from *Ae. speltoides*/ wheat substitution lines (Kota and

Dvořák, 1988). The wide hybridization between bread wheat and rye (*Secale cereale*) has been successfully used in wheat breeding programs. Several wheat genomic rearrangements, chromosome instabilities and structural variations of wheat chromosomes were detected in wheat/rye derived lines such as the monosomic and disomic rye addition lines (Bento *et al.,* 2010, Fu *et al.,* 2013). In addition, karyotype analysis of the octoploid triticale lines derived from crosses of bread wheat to rye followed by a backcross to the bread wheat parent (Tang *et al.,* 2014), using mc-FISH using Oligo-pSc119.2-1, Oligo-pTa535-1 probes and GISH using rye genomic DNA as probes, showed alterations in wheat chromosomes including the 5A, 6A, 1B, 2B, 6B, 7B, 1D, 3D and 7D chromosomes. Two intra-genomic rearrangements 1BS-2BS and 1BL-2BL chromosomes were identified in one of the lines (Tang *et al.,* 2014). Moreover, intergenomic rearrangements between wheat A, B and D-genomes were found in advance backcross lines of wheat/*Am. muticum* introgression lines (King *et al.*, 2017). These studies indicate that the structure of wheat chromosomes could be changed in lines derived from wide hybridization. Although abundant genetic diversity was stored in wheat-alien hybrids, the structural alterations of wheat chromosomes can form a new source of variations that can be used in wheat breeding.

5.1.4 Intergenomic rearrangements identified in wheat/*Am. muticum* introgression lines

Amblyopyrum muticum [(Boiss.) Eig. (*Aegilops mutica* Boiss.) (2n=2x=14; genome TT)] is an annual wild relative of wheat. It is a native species in Turkey and Armenia (Kilian *et al.,* 2013) *Am. muticum* was reported to be resistant against environmental stresses (Iefimenko *et al.,* 2015) and fungal diseases such as powdery mildew (Eser, 1998) and leaf rust (Dundas *et al.,* 2015). *Am. muticum* introgression into bread wheat is an ongoing project in our research group at the University of Nottingham, part of a research program with the objective of the transfer of genetic variation for agronomically important traits from wild and distantly related species into wheat using a whole genome introgression approach (King *et al.,* 2013, 2017). As described by King *et al.* (2017), for the generation of wheat/*Am. muticum* introgression lines, a total of 1039 crosses (crossed ears) were made between *Am. muticum* and Paragon.

(**Figure 5-1**) resulting in the production of 8146 F_1 hybrid seeds. As a result of the low germination rate (28.6%) and fertility of the F_1 hybrids, only 34 BC_1 individuals were generated after backcrossing to Paragon, of which only 16 plants grew to maturity and set seed. The 35K Axiom HD Wheat-Relative Genotyping array was used to detect wheat/wild relative introgressions in backcross progenies enabling the detection and characterization of 218 genome wide wheat/*Am. muticum* introgressions (King *et al.*, 2017).

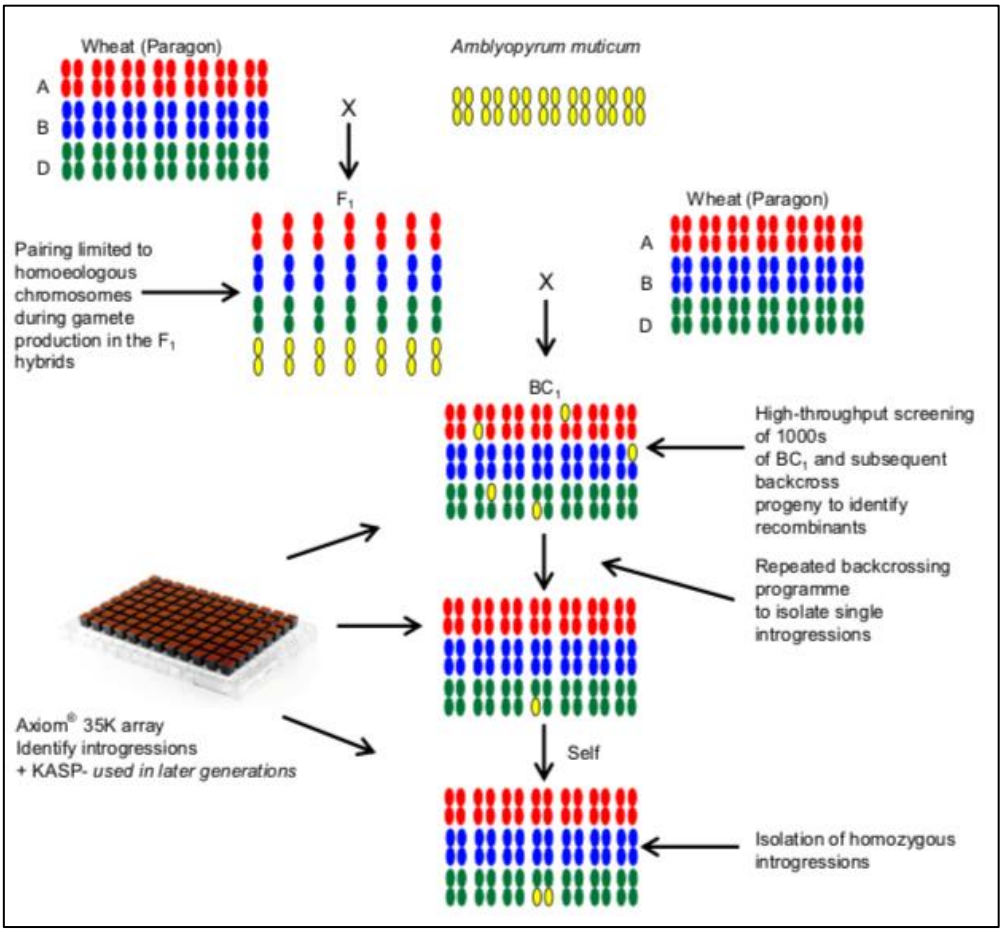

Figure 5-1. Wheat/*Am. muticum* introgression strategy (King *et al.*, 2017).

To confirm the SNP analysis, genotyped BC_3 individuals were selected and analysed by mc-GISH that not only confirmed the presence of the *Am. muticum* segment and/or introgression but also revealed the presence of intergenomic recombinant events between the A, B and D genomes of wheat (King *et al.*, 2017). These hexaploid wheat/alien introgression lines harbouring genomic rearrangement involving the D-genome with either the A-or the B-genome, could be an alternative strategy for D-genome introgression into durum wheat through pentaploid crosses.

The hypothesis behind this chapter is the use of a novel crossing strategy to introgress the D-genomic segment present hexaploid wheat/*Am muticum* introgression lines harbouring D-genomic translocation into durum wheat, in either the presence or absence of *Am. muticum* segments.

5.2 Material and methods

5.2.1 Plant material

Seed produced (self or backcrossed) from eight BC_3-F_1 wheat/*Am. muticum* introgression lines carrying translocations involving the D-genome with either the A- or B-genomes or both, and two durum wheat genotypes "Karim" and "Om Rabiaa 5" were used as parental lines in this study. Three of the BC_3-F_1 wheat/*Am. muticum* introgression lines used had one to three T-genome segments of *Am. muticum* (King *et al.*, 2017) (**Table 5-1**). Self or back crossed seed of each BC_3-F_1 wheat/*Am. muticum* introgression lines, designated as WMI (wheat/*muticum* introgression) lines, were used in the present study.

Table 5-1. Type and number of the D- genomic translocations and T-genome segments present in the parental introgression lines and the reference of the WMI lines used in the crosses.

Group	Parental lines ref.	Genomic translocation*$^{No.}$	No. of T-genome segments	WMI lines used to cross
G1	**BC_3-F_1-157-C**	A-d*^{1}	0	BC_4-F_1-129
	BC_3-F_1-157-D	D-a*^{1}	0	BC_4-F_1-130
	BC_3-F_1-157-E	A-d*^{1}	0	BC_3-F_2-130
	BC_3-F_1-172-C	D-a*^{1}	0	BC_3-F_2-132
G2	**BC_3-F_1-172-E**	D-a-b*^{1} + A-d*^{1}	3	BC_3-F_2-133
	BC_3-F_1-177-E	D.a-b*^{1} + A.D*^{1}	2	BC_3-F_2-134
	BC_3-F_1-244-A	d-A-d*^{1} + D-a*^{1}	1	BC_3-F_2-135
	BC_3-F_1-244-B	A-d*^{1}	2	BC_3-F_2-136

NB: *$^{No.}$ indicates the number of copies, G1= WMI parental lines without T-genome segment, G2= WMI parental lines carrying T-genome segments.

5.2.2 Crossing plan

Two to four WMI lines, depending on the number of seed available and the viability of the seeds, were germinated. In total, 28 WMI seeds were germinated at 22°C for 2–3 days and plants were grown to maturity in glasshouse conditions

at 25°C with a photoperiod of 12 hours after vernalisation for four weeks. Three replicates of 12 durum wheat plants from each genotype were planted at two week intervals. WMI lines were crossed as the female to the durum wheat as described in section 2.2. The F_1 seeds produced from each combination were then backcrossed to the recurrent durum wheat parent as a female parent. The BC_1-F_1 plants were allowed to self-pollinate to advance generations. All the plants were screened for the presence of the D-genomic translocations at every generation. Only the lines carrying D-genomic translocations were allowed to advance to the next generation (**Figure 5-2**). To follow the lines produced, the parental WMI lines were divided into two groups (**Table 5-1**). The first group (G1) includes the parental lines that carried only wheat sub-genomic rearrangements (no *Am. muticum* introgressions), whereas the second group (G2) included the parental lines that retained one to three T-genome segments (**Table 5-1**) (King *et al.*, 2017).

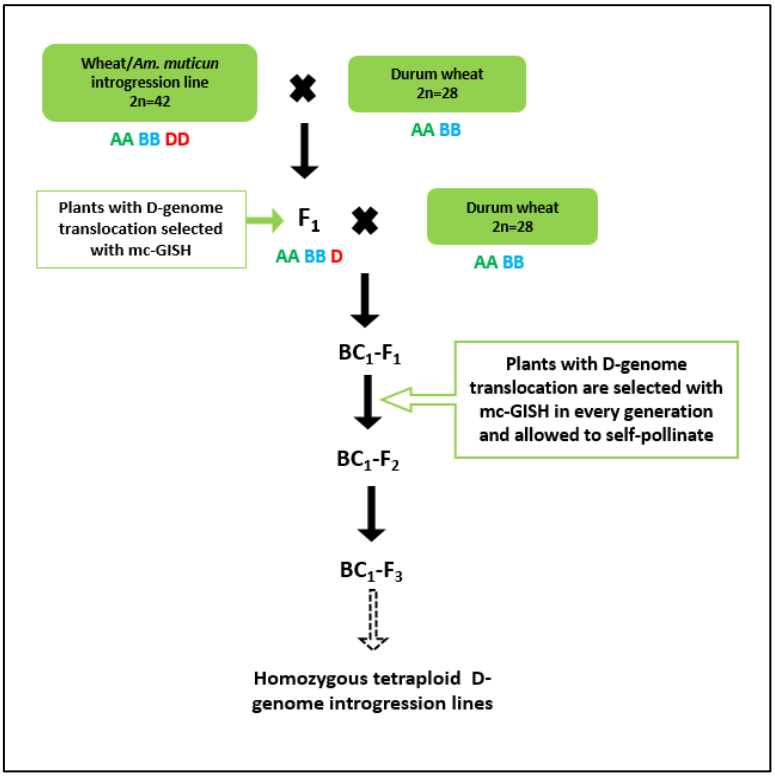

Figure 5-2. The crossing diagram of the introgression of the D- chromosome translocations identified in wheat/*Am. muticum* introgression lines into durum wheat.

5.2.3 Cytogenetic analysis

5.2.3.1 Single colour and multicolour GISH analysis

Progeny produced out of the crossing diagram (**Figure 5-2**) were analysed using mc-GISH for identification of the D-genomic translocations. Single colour-GISH using labelled *Am. muticum* genomic DNA as a probe was used to detect the presence of the T-genome segment(s) in selected advanced generations. The GISH protocol was applied as described in section 2.5.

5.2.3.2 Multicolour-FISH analysis

Metaphase spreads of some selected lines in the BC_1-F_3 generation were analysed via mc-FISH using the two repetitive DNA sequences pSc119.2 and pAs.1 as probes labelled by nick translation with Chroma Tide Alexa Fluor 488-5-dUTP (green) and Alexa Fluor 594-5-dUTP (red) respectively, and counterstained by DAPI (bleu) as described in section 2.5.

5.3 Results

5.3.1 The F_1 generation

5.3.1.1 F_1 seed production

In total, 32 self or backcrossed seed of the eight parental WMI lines were sown. Out of 28 seeds sown, 24 seed germinated and reached maturity (**Table 5-2**). Every WMI line was crossed to both of the durum wheat varieties "Om Rabiaa 5" and "Karim".

Table 5-2. Germination of the WMI lines used in the F_1 production.

Group	WMI lines crossed	No. of seed sown	No. of seed germinated	No. of plants reaching maturity
G1	**BC_4-F_1-129**	4	4	4
	BC_4-F_1-130	4	3	1
	BC_3-F_1-130	6	4	4
	BC_3-F_2-132	4	3	2
G2	**BC_3-F_2-133**	4	4	4
	BC_3-F_2-134	2	2	2
	BC_3-F_2-135	4	4	3
	BC_3-F_2-136	4	4	4
Total		**32**	**28**	**24**

In total, 63 crosses were made of which 35 crosses were with "Karim" and 28 with "Om Rabiaa 5" (**Table 5-3**). All of the crosses made with "Om Rabiaa 5" set seeds, allowing the production of 246 F_1 hybrid seeds. However, only 29 crosses (82%) with "Karim" set seed giving rise to 242 F_1 hybrid seeds. The average seed set per cross using "Om Rabiaa 5" was also higher than when using "Karim", indicating a higher compatibility of the WMI lines with "Om Rabiaa 5" compared to "Karim" (**Table 5-3**).

Table 5-3. The production of the F_1 hybrids.

F_1 Cross-combination	No. of crosses	No. of crosses that set seed	% of crosses that set seed	No. of F_1 seed produced	Average seed set/cross
WMI lines /Om Rabiaa 5	28	28	100%	246	8.78
WMI lines /Karim	35	29	82%	242	6.91
Total	**63**	**57**	**90%**	**488**	**7.74**

5.3.1.2 Cytogenetic analysis of the F_1 lines

One to six F_1 hybrid seeds, depending on the availability of seed were randomly selected from 20 crosses with "Karim" and another 20 with "Om Rabiaa 5". **Table 3-3** summarises the seed sown and germinated. A higher germination rate was registered for the seed of the crosses made with "Karim". In total, the 104 F_1 hybrid lines were screened using mc-GISH for the presence of genomic translocations involving the D-genome with either the A or/and B-genome. Overall, only 33.6% (36 lines) of the lines showed the presence of genomic translocations. On average, the percentage of the retention of the genomic translocation was higher using "Karim" (35%) compared to "Om Rabiaa 5" (32%).

After crossing the hexaploid WMI lines (2n=42) to the durum wheat, pentaploid F_1 lines with 35 chromosomes were expected. However, the chromosome number varied between 31 and 35 in both of the cross-combinations. The number of univalent D-genome chromosomes retained varied from four to six. In addition, different genomic translocations involving the D-genome with either the A, B or both were identified. Similar to the translocation notation system used in the chapter 4, genomic translocations were designed by the letter of the genome involved (A, B or D). Upper case letter

designated the largest segment, whereas lower case letter designated the small segment. In paracentric translocations, the two letters are separated by a dash (e.g. A-d), whereas for centromeric translocations, a dot is used (e.g. A.B). Some examples of the translocations found are represented in **Figure 5-3**.

Table 5-4. The F_1 hybrid seed germination and its screening for the D-genomic translocations.

Cross combination	No. of crosses selected	No. of F_1 Seed sown	No. of F_1 seed germinated	% of germination	No. of F_1 plants with a translocation	% of lines with a translocation
WMI lines /Om Rabiaa 5	20	78	50	64%	16	32%
WMI lines /Karim	20	71	54	76%	19	35%
Total/average	**40**	**149**	**104**	**70%**	**35**	**33.6%**

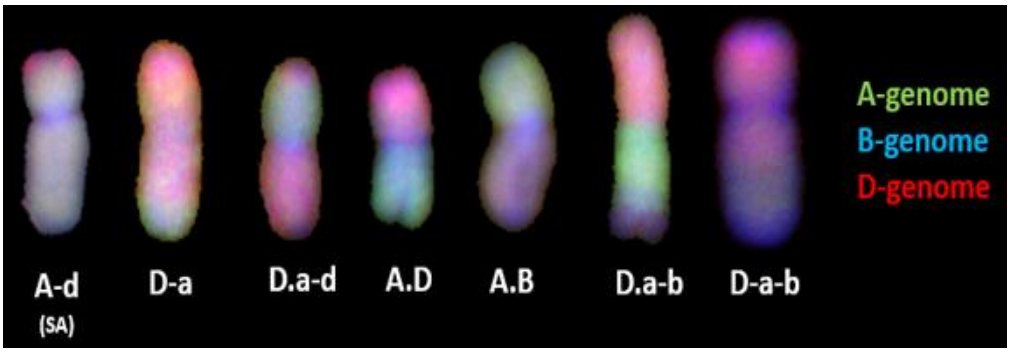

Figure 5-3. Mc-GISH showing the different chromosome translocations identified in the F_1 progeny.

5.3.1.3 The screening of the F_1 lines of the crosses with "Om Rabiaa 5"

Mc-GISH showed that 69% of the F_1 lines (11 lines) retained at least one of the translocations present in the parental line. For example, only one of the two translocations D-a-b and A-d present in two of the original parental was retained in the F_1 lines OR-14-C, OR-19-A and OR-17-C, while one of the F_1 hybrid lines, F_1-OR-7-B, contained two A-d translocation type (**Figure 5-4a**). Five lines showed the presence of one, two or three new genome translocations involving either the A- and the D-genome or the A and the B-genome (underlined translocations in **Table 5-5**). The simultaneous presence of AS.BL and BS.AL translocations in two of the lines indicate the occurrence of chromosome arm exchange between an A- and a B-genome chromosome during meiosis (**Figure 5-4b**). Not all 16 lines showing a genome translocation reached

maturity. In fact, 25% of the lines described in **Table 5-5** failed to reach the maturity stage.

Table 5-5. Genomic composition and translocation type of the F_1 hybrid lines of the crosses with "Om Rabiaa 5".

Grp.	Line No.	F_1 lines reference	F_1 pedigree	A-, B- and D-genome chrom. No.	Translocation type$^{*No.}$	Total chrom. No.	MS
G1	1	**F_1-OR-1-A**	BC_4-F_1-129-A/ Om Rabiaa 5	14A+14B+5D	A-d^{*1} (SA)	34	Y
	2	**F_1-OR-1-B**	BC_4-F_1-129-A/ Om Rabiaa 5	14A+14B+5D	A-d^{*1} (SA)	34	Y
	3	**F_1-OR-4-B**	BC_4-F_1-129-D/ Om Rabiaa 5	14A+14B+4D	A-d^{*1} (SA)	33	Y
	4	**F_1-OR-5-C**	BC_4-F_1-130-D/ Om Rabiaa 5	14A+14B+6D	D-a^{*1}	35	Y
	5	**F_1-OR-7-B**	BC_3-F_2-130-B/ Om Rabiaa 5	13A+14B+6D	A-d^{*2} (SA)	35	Y
	6	**F_1-OR-11-A**	BC_3-F_2-132-D/ Om Rabiaa 5	14A+14B+6D	D-a^{*1}	35	Y
G2	7	**F_1-OR-14-C**	BC_3-F_2-133-D/ Om Rabiaa 5	14A+14B+6D	D-a-b^{*1}	35	Y
	8	**F_1-OR-19-A**	BC_3-F_2-133-D/ Om Rabiaa 5	14A+14B+6D	D-a-b^{*1}	35	Y
	9	**F_1-OR-17-C**	BC_3-F_2-133-C/ Om Rabiaa 5	14A+13B+6D	D-a-b^{*1}	34	Y
	10	**F_1-OR-23-A**	BC_3-F_2-135-C/ Om Rabiaa 5	14A+14B+5D	D.a-d^{*1}	34	N
	11	**F_1-OR-24-A**	BC_3-F_2-135-D/ Om Rabiaa 5	14A+14B+4D	A.D^{*2}	34	N
	12	**F_1-OR-24-B**	BC_3-F_2-135-D/ Om Rabiaa 5	14A+14B+4D	A.D^{*1}	33	N
	13	**F_1-OR-25-B**	BC_3-F_2-136-B/ Om Rabiaa 5	13A+13B+6D	d-A-d^{*1} + A.B^{*2}	35	Y
	14	**F_1-OR-27-C**	BC_3-F_2-136-C/ Om Rabiaa 5	13A+13B+6D	D-a^{*1} + A.B^{*2}	35	Y
	15	**F_1-OR-28-A**	BC_3-F_2-136-D/ Om Rabiaa 5	14A+14B+6D	A-d^{*1} (SA)	35	N
	16	**F_1-OR-28-C**	BC_3-F_2-136-D/ Om Rabiaa 5	14A+13B+6D	A-d^{*1} (SA)	34	Y

NB: MS=Maturity stage, Y=yes, N=no, SA=translocation on the short arm, $^{*No.}$ = indicates the number of copies; +t indicates the presence of a telomere, G1= WMI parental lines without T-genome segment, G2= WMI parental lines carrying T-genome segments.

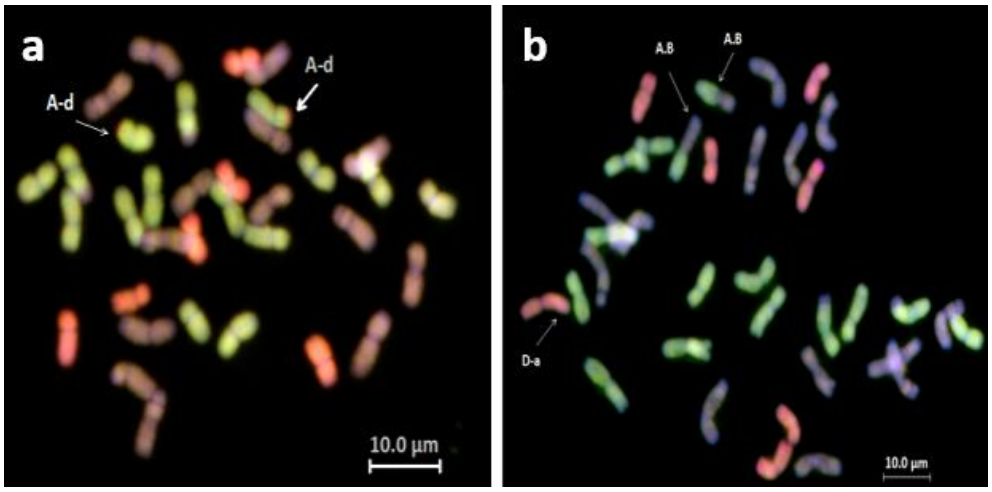

Figure 5-4. Mc-GISH of a root-tip metaphase spread of (a) F_1-OR-7-B and (b) F_1-OR-27-C showing the presence of translocations (Chromosome colour-code: A: green, B: purple, D: red).

5.3.1.4 The screening of the F_1 lines of the crosses with "Karim"

Mc-GISH analysis showed that 95% of the F_1 hybrid lines of the crosses made with "Karim" had retained at least one copy of the parental translocation (**Table 5-6**). Seven F_1 sister lines (F_1-KR-18-A to G) had retained both the A.D and the D.a-b translocations. In fact, mc-GISH analysis of their parental line BC_3-F_2-134-B, used in the crossing with "Karim", showed the presence of homozygous copies of the D-genomic translocations, which facilitated their retention in all the F_1 hybrid lines screened. Furthermore, four lines showed the presence of new genomic translocations alongside the retained ones. One line, F_1-KR-26-B, previously carrying a telomeric A-d translocation, showed the presence instead of d-A-d translocation. This can be explained by the occurrence of new D-translocation in the same translocated A-genome chromosomes in the telomeric region of the other arm. Overall, 15 % of the lines identified with genome translocations failed to reach the adult stage.

Table 5-6. Genomic composition of the F_1 of "WMI lines /Karim" crosses carrying D-genomic translocations.

Grp.	Line No.	F_1 lines reference	F_1 pedigree	A-, B- and D-genome chrom. No.	Translocation type$^{*No.}$	Total chrom. No.	MS
G1	1	**F_1-KR-1-A**	BC_4-F_1-129-A/ Karim	14A+14B+6D	A-d^{*1}(SA)	35	Y
	2	**F_1-KR-1-D**	BC_4-F_1-129-A/ Karim	14A+14B+5D	A-d^{*1}(SA)	34	Y
	3	**F_1-KR-5-A**	BC_4-F_1-130-A/ Karim	14A+12B+6D	D-a^{*1}	33	Y
	4	**F_1-KR-5-B**	BC_4-F_1-130-A/ Karim	14A+12B+6D	D-a^{*1}	33	Y
	5	**F_1-KR-7-B**	BC_3-F_2-130-B/ Karim	14A+13B+6D	A-d^{*1}(SA)	34	Y
	6	**F_1-KR-10-A**	BC_4-F_1-130-B/ Karim	14A+12B+6D	D-a^{*1}	33	Y
G2	7	**F_1-KR-18-A**	BC_3-F_2-134-B/ Karim	12A+14B+6D	D.a-b^{*1} + A.D^{*1}	34	Y
	8	**F_1-KR-18-B**	BC_3-F_2-134-B/ Karim	12A+14B+5D	D.a-b^{*1} + A.D^{*1}	33	Y
	9	**F_1-KR-18-C**	BC_3-F_2-134-B/ Karim	12A+14B+5D	D.a-b^{*1} + A.D^{*11}	33	Y
	10	**F_1-KR-18-D**	BC_3-F_2-134-B/ Karim	12A+14B+6D	D.a-b^{*1} + A.D^{*1}	34	Y
	11	**F_1-KR-18-E**	BC_3-F_2-134-B/ Karim	12A+14B+5D	D.a-b^{*1} + A.D^{*1}	33	Y
	12	**F_1-KR-18-F**	BC_3-F_2-134-B/ Karim	12A+14B+5D	D.a-b^{*1} + A.D^{*1}	33	Y
	13	**F_1-KR-18-G**	BC_3-F_2-134-B/ Karim	12A+14B+5D	D.a-b^{*1} + A.D^{*1}	33	Y
	14	**F_1-KR-19-A**	BC_3-F_2-135-B/ Karim	14A+14B+5D	D-a^{*1} + <u>D-a-b^{*1}</u>	35	Y
	15	**F_1-KR-22-A**	BC_3-F_2-135-D/ Karim	12A+14B+4D	A-d^{*1}(SA) +<u>A.D^{*2}</u>	33	N
	16	**F_1-KR-26-A**	BC_3-F_2-136-B/ Karim	14A+13B+5D	D-a^{*1} + <u>B.D^{*1}</u>	34	N
	17	**F_1-KR-26-B**	BC_3-F_2-136-B/ Karim	14A+14B+6D	<u>d-A-d^{*1}</u>	35	Y
	18	**F_1-KR-28-B**	BC_3-F_2-136-B/ Karim	14A+14B+6D	A-d^{*1}(SA)	35	Y
	19	**F_1-KR-29-A**	BC_3-F_2-136-D/ Karim	14A+14B+6D	A-d^{*1}(SA)	35	N

NB: MS=Maturity stage, Y=plant reached Maturity stage, N=Plant didn't reach maturity stage, $*^{No.}$ =indicates the number of copies; +t indicates the presence of a telomere, G1= WMI parental lines without T-genome segment, G2= WMI parental lines carrying T-genome segments.

5.3.2 The BC_1-F_1 generation

5.3.2.1 BC_1-F_1 seed production

With the aim of reducing the chromosome number to 28 chromosomes and to obtain a single genomic translocation per line, all of the F_1 lines that reached maturity from the two cross-combinations with "Karim" or "Om Rabiaa 5" were backcrossed to the respective recurrent durum wheat parent. A total of 68 backcrosses were made, i.e. 31 and 37 with "Om Rabiaa 5" and "Karim", respectively. Results showed that a higher percentage of crosses set seed (67%) and a higher average seed set per cross (7 seeds/cross) were registered with the "Om Rabiaa 5" genotype compared to "Karim" (54% of crosses set seed; 5 seed/cross) (**Table 5-7**). In addition, the average seed set per cross produced at the F_1 and the BC_1 generations, showed that a higher seed set was obtained using the "Om Rabiaa 5" genotype compared to "Karim" (**Figure 5-5**).

Table 5-7. The BC_1-F_1 seed production.

BC_1-Cross combination Pedigree	No. of crosses	No. of crosses that set seed	Percentage of crosses setting seed	No. of F_1 seed produced	Average seed set/cross
WMI lines /Om Rabiaa 5*2	31	21	67%	149	7
WMI lines/Karim*2	37	20	54%	105	5
Total/Average	**68**	**41**	**60%**	**254**	**6**

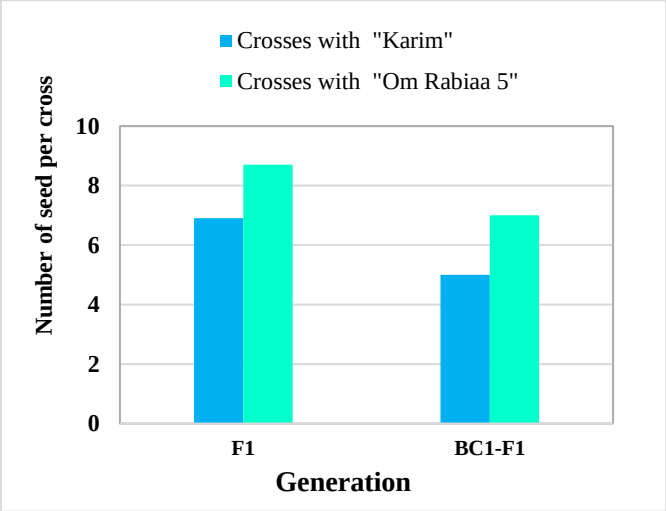

Figure 5-5. Average seed set number per cross in the F_1 and the BC_1 generation in the two cross-combinations with "Karim" and "Om Rabiaa 5" genotypes.

5.3.2.2 Cytogenetic analysis of the BC_1-F_1 lines

Depending on the seed number obtained per cross as well as the number of translocations present per line, nine BC_1-F_1 crosses "Om Rabiaa 5" and other 11 BC_1-F_1 crosses with "Karim" (**Table 5-8**). A total of 74 seeds were sown of which only 64 germinated, with a slightly higher germination rate (87%) of the seed belonging to the crosses with "Om Rabiaa 5" compared to "Karim" (85%). Mc-GISH analysis showed the presence of genomic translocations in 36% of these lines. More lines with genomic translocations (some involving the D genome with either the A or B genomes and others involving the A and B genomes) were identified in the back-ground of the crosses with "Karim" (13 lines) compared to the ones with "Om Rabiaa 5" (10 lines) (**Table 5-8**).

Table 5-8. The BC_1-F_1 seed germination and screening for D-genomic translocations.

BC_1-F_1 Cross-combination	No. of crosses selected	No. of Seed sown	No. of seed germinated	% of germination	No. of plants with translocation	% of lines with translocation
WMI lines/ Om Rabiaa 5*2	9	32	28	87%	10	35%
WMI lines/ Karim*2	11	42	36	85%	13	36%
Total	**20**	**74**	**64**	**86%**	**23**	**36%**

5.3.2.3 The screening of the BC_1-F_1 lines of the crosses with "Om Rabiaa 5"

The genomic composition of the ten BC_1-F_1 lines with genomic translocations in the crosses using "Om Rabiaa 5", showed a considerable reduction in the D-genome chromosome retention and consequently in the total chromosome number after backcrossing with the exception of one line, that had lost only one D-genome chromosome out of five (**Table 5-9**). Cytogenetic analysis showed that all lines retained at least one copy of the translocation identified in their F_1 parental lines before backcrossing. Two lines had lost all of the monosomic D-genome chromosomes and retained an A-d and a D-a-b genomic translocation respectively, as an addition in a total of 29 chromosomes (**Figure 5-6**). One line had lost all of the D-genome chromosomes and retained an A-d telomeric translocation (a small D-genome segment in the short arm of an A-genome chromosome as a substitution of an A-genome chromosome), in a total of 28 chromosomes (**Figure 5-6**). Both of the A.B translocations

previously described in F_1-OR-25-B and F_1-OR-27-C were retained in the BC_1-F_1s after backcrossing. Three of the lines carrying translocations failed to reach maturity.

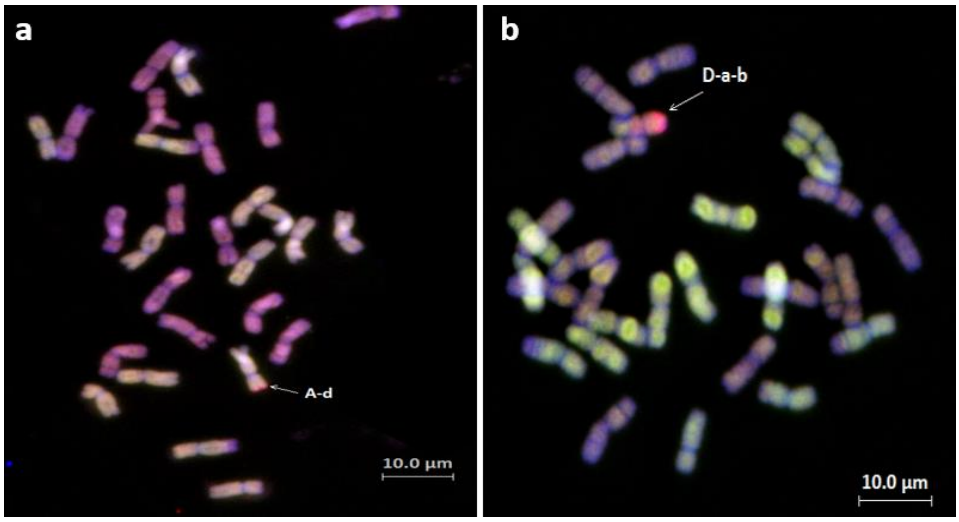

Figure 5-6. Mc-GISH of root-tip metaphase spreads of (a) BC_1-F_1-OR-7-B-A and (b) BC_1-F_1-OR-19-A-C showing an A-d and D-a-b translocation, respectively (Chromosome colour-code: A: green, B: purple, D: red).

Table 5-9. Genomic composition and translocation type of the BC_1-F_1 hybrid lines of the crosses with "Om Rabiaa 5".

Grp.	Lines No.	F_1 lines reference	A-, B- and D-genome chrom. No.	Translocation type$^{*No.}$	Total chrom. No.	No. of seed produced
G1	1	**BC_1-F_1-OR-1-A-B**	14A+14B	A-d*^{1}(SA)	29	2
	2	**BC_1-F_1-OR-5-C-A**	14A+14B+2 D	D-a*^{1}	31	20
	3	**BC_1-F_1-OR-7-B-A**	13A+14B	A-d*^{1}(SA)	28	79
	4	**BC_1-F_1-OR-7-B-D**	13A+14B+3 D	A-d*^{1}(SA)	31	7
G2	5	**BC_1-F_1-OR-14-C-A**	14A+13B+2 D	D-a-b*^{1}	30	S
	6	**BC_1-F_1-OR-19-A-C**	14A+14B	D-a-b*^{1}	29	14
	7	**BC_1-F_1-OR-25-B-A**	14A+13B+3 D	A-d*^{1}(SA) + A.B*^{2}	33	N
	8	**BC_1-F_1-OR-25-B-B**	14A+15B+5 D	A-d*^{1}(SA)	35	24
	9	**BC_1-F_1-OR-27-C-A**	13A+13B+3 D	D-a*^{1}+ A.B*^{2}	32	N
	10	**BC_1-F_1-OR-28-C-A**	14A+14B+2 D	A-d*^{1}(SA)	31	12

N = Plant didn't reach maturity stage, S = Sterile, *$^{No.}$ =indicates the number of copies, G1= WMI parental lines without T-genome segment, G2= WMI parental lines carrying T-genome segments.

5.3.2.4 Screening of BC_1-F_1 lines of the crosses with "Karim"

A considerable reduction in the chromosome number was noticed in the BC_1-F_1 progeny of the crosses with "Karim" induced by the loss of most of the whole D-genome chromosomes in all of the lines. In fact, D-genome chromosome retention decreased to only one to 3 chromosomes. In comparison to the BC_1 generation, mc-GISH analysis of the 13 BC_1-F_1 lines showed that 69% of the lines (nine lines) had retained at least one copy of the translocation identified in the original F_1 (**Table 5-10**).

In total, ten new translocations were counted (mostly translocations between the D genome with either the A- or B-genomes but also one translocation between the A and B genomes). Besides one A.B RobT, all the translocations were telomeric involving the translocation of a small D-genome segment in the telomeric region of an A or a B-genome chromosome. Two lines showed the presence of three genome translocations of which two translocations (A.D and D.a-b) were retained from the previous generation and one was a new A-d translocation in the telomeric region of the long arm of an A-genome chromosome (e.g.**Table 5-7**). One line had lost all of the previous translocations and contained a single new A-d (LA) translocation type substituting an A-genome chromosome in a total of 28 chromosomes. While 54% of the BC_1-F_1 lines produced BC_1-F_2 seed, the remaining 46% of the lines were either sterile or failed to reach maturity.

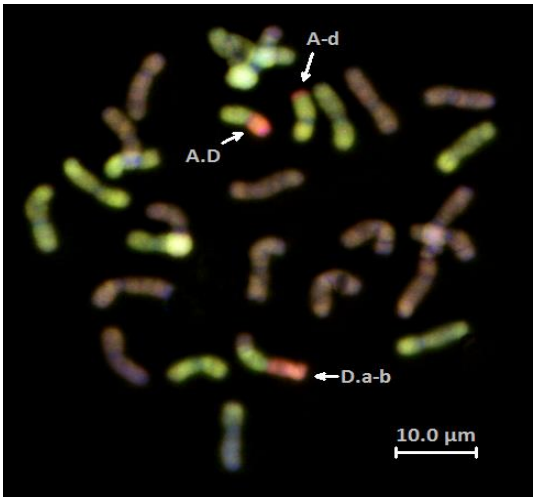

Figure 5-7. Mc-GISH of root-tip metaphase spread of BC_1-F_1-KR-18-A-D showing three D-genomic translocations in a total of 29 chromosomes (Chromosome colour-code: A: green, B: purple, D: red).

Table 5-10. Genomic composition and translocation type of the BC_1-F_2 lines of the crosses with "Karim".

Grp	Line No.	BC_1-F_1 lines reference	A-, B- and D-genome chrom. No.	Translocation type*No.	Total Chrom. No.	BC_1-F_2 seed produced
G1	1	BC_1-F_1-KR-7-B-C	14A+14B+1D	A-d*1 (SA)	30	20
G2	2	BC_1-F_1-KR-18-A-A**	-	A-d*1(LA) + A.B*1	-	18
	3	BC_1-F_1-KR-18-A-C	13A+14B	A-d*1 (SA)	28	82
	4	BC_1-F_1-KR-18-A-D	12A+14B	D-a-b*1 + A.D*1 + A-d*1(LA)	29	62
	5	BC_1-F_1-KR-18-C-A**	-	D-a-b*1 + A.D*1 + A-d*1(LA)	-	10
	6	BC_1-F_1-KR-18-C-D	14A+13B+2D	D-a-b*1 + A-D*1	31	N
	7	BC_1-F_1-KR-18-D-A	11A+14B+3D	D-a-b*1 + B-d*1(SA)	30	N
	8	BC_1-F_1-KR-18-D-B	15A+14B+1D	A-D*1	31	S
	9	BC_1-F_1-KR-18-E-B	13A+14B+1D	A-d*1(SA)	29	S
	10	BC_1-F_1-KR-18-G-B	14A+13B+1D	D.a-b*1	29	S
	11	BC_1-F_1-KR-19-A-B	14A+14B	d-A-d*1	29	50
	12	BC_1-F_1-KR-19-A-C	14A+14B+2D	d-A-d*1	31	45
	13	BC_1-F_1-KR-28-B-C	14A+14B+1D	D-a*1 + B-d*1(LA)	30	N

NB: *No. indicates the number of copies, N=Plant didn't reach maturity, S=sterile, **unclear GISH pictures, G1= WMI parental lines without T-genome segment, G2= WMI parental lines carrying T-genome segments.

5.3.3 Production and mc-GISH screening of the BC_1-F_2 generation for the retention of the D-genomic translocation

Only 13 BC_1-F_1 lines gave rise to BC_1-F_2 seeds of which six were in the cross combination with "Om Rabiaa 5" and seven in the crosses with "Karim" (**Table 5-10**). Out of 88 BC_1-F_2 seed sown, 82 germinated showing a considerable improvement in the germination rate (93%) compared to the previous generations (**Table 5-11**). Mc-GISH analysis revealed that around 40% of the 82 BC_1-F_2 lines had at least one translocation involving the D-genome. As seen in the BC_1-F_1 generation, more translocations were found in the BC_1-F_2 progeny

of the crosses with “Karim (42.5%) compared to the one with “Om Rabiaa 5” (30.2%).

Table 5-11. Germination and screening of the BC_1-F_2 for D-genomic translocations.

BC_1-F_2 Cross-combination pedigree	No. of crosses selected	No. of Seed sown	No. of seed germinated	Percentage of germination	No. of plants with translocation	% of lines with translocation
WMI lines/ Om Rabiaa 5	6	46	43	93.45%	13	30.2%
WMI lines/ Karim	7	50	47	94%	20	42.5%
Total	**13**	**88**	**82**	**93.1%**	**33**	**40.2%**

5.3.3.1 Mc-GISH analysis of the BC_1-F_2 lines in the crosses with “Om Rabiaa 5”

Out of the six BC_1-F_1 lines in the crosses with “Om Rabiaa 5” that set BC_1-F_2 seed, translocations were found in the self-progeny of only two of the BC_1-F_1 lines (**Table 5-12**). BC_1-F_1-OR-7-B-A contained a single A-d translocation introgressed into 28 chromosomes. Out of eight BC_1-F_2 seed screened, six of the plants had a homozygous D-introgression in a tetraploid background of “Om Rabiaa 5” after the retention of two copies of the A-d translocation in a total of 28 chromosomes (**Table 5-12**). This translocation, initially present in the BC_3-F_2-130 parental line in the absence of a T-genome segment, was successfully introgressed into an “Om Rabiaa 5” background. All six of the lines with homozygous D-introgressions had a durum wheat head type (e.g. **Figure 5-8**). These lines were fertile and set good numbers of BC_1-F_3 seed (**Table 5-12**).

Figure 5-8. (a) Plant, (b) spike morphology and (c) mc-GISH of a root-tip metaphase spread of the tetraploid BC_1-F_2-OR-7-B-A-G showing a homozygous D-genome introgression (Chromosome colour-code: A: green, B: purple, D: red).

Table 5-12. Selected BC_1-F_2 lines of "WMI lines/Om Rabiaa 5" carrying D-genome translocations.

Grp	Line No.	BC_1-F_2 lines reference	A-, B- and D-genome chrom. No.	Translocation type*No.	No. T-genome segments	Total Chrom. No.	No. of BC_1-F_3 seed produced
G1	1	**BC_1-F_2-OR-7-B-A-A**	12A+14B	A-d*1(SA)	0	28	67
	2	**BC_1-F_2-OR-7-B-A-C**	12A+14B	A-d*2(SA)	0	28	110
	3	**BC_1-F_2-OR-7-B-A-D**	12A+14B	A-d*2(SA)	0	28	103
	4	**BC_1-F_2-OR-7-B-A-G**	12A+14B	A-d*2(SA)	0	28	147
	5	**BC_1-F_2-OR-7-B-A-H**	12A+14B	A-d*2(SA)	0	28	146
	6	**BC_1-F_2-OR-7-B-A-E**	12A+14B	A-d*2(SA)	0	28	20
G2	7	**BC_1-F_2-OR-25-B-B-A**	15A+16B+6D	A-d*1(SA) + D-a*1	2	39	S
	8	**BC_1-F_2-OR-25-B-B-B**	14A+14B+6D	d-A-d*1 + D.a-d*1	2	38	1
	9	**BC_1-F_2-OR-25-B-B-C**	14A+14B+2D	B.D*1 + A.D*1	1	32	S
	10	**BC_1-F_2-OR-25-B-B-D**	14A+14B+1D	D.a-d*1 + B.D*1 + A.D*1	0	32+t	S
	11	**BC_1-F_2-OR-25-B-B-F**	13A+14B+3D	B.D*1 + B-A-d*1	1	32	S
	12	**BC_1-F_2-OR-25-B-B-G**	14A+15B+4D	A-d*1(SA) + D-a*1 + B-d*1	1	36	S
	13	**BC_1-F_2-OR-25-B-B-I**	11A+13B+2D	A-d*1(SA) + B.D*1 + B-a-d*1	0	29+2t	S

NB: *No. indicates the number of copies; +t indicates the presence of a telomere, MS=Maturity stage, Y=yes, N=no, S=sterile, G1= WMI parental lines without T-genome segment, G2= WMI parental lines carrying T-genome segments.

The screening of the nine BC_1-F_2 selfed lines of the BC_1-F_1-OR-25-B-B line, revealed the presence of genomic translocations in seven of the lines with whole D-genome chromosome retention varying between one to six chromosomes. In comparison to the parental BC_1-F_1-OR-25-B-B lines, only three lines had retained the A-d (SA) translocation. In addition, all of the seven lines showed the presence of one to three new genomic translocations involving the D-genome with the A- or the B-genome as well as translocations involving the three genomes at the same time (underlined translocation; **Table 5-12**, e.g. **Figure 5-9a**). The parental line used to generate the F_1 hybrid belonged to G2, i.e. it contained an *Am. muticum* segment), BC_1-F_1-OR-25-B-B and the seven

BC_1-F_2 progeny lines were screened for the presence of a T-genome segment via sc-GISH. The sc-GISH revealed the presence of one *Am muticum* introgression in BC_1-F_1-OR-25-B-B line translocated with a B-genome chromosome, which was then retained in five of the BC_1-F_2 lines screened, either as a single copy in three lines or two copies in two of the lines (e.g. **Figure 5-9b**). All of the lines were sterile when self-pollinated and didn't set seed except BC_1-F_2-OR-25-B-B-B, which showed a very low fertility level in which only one BC_1-F_3 seed was found.

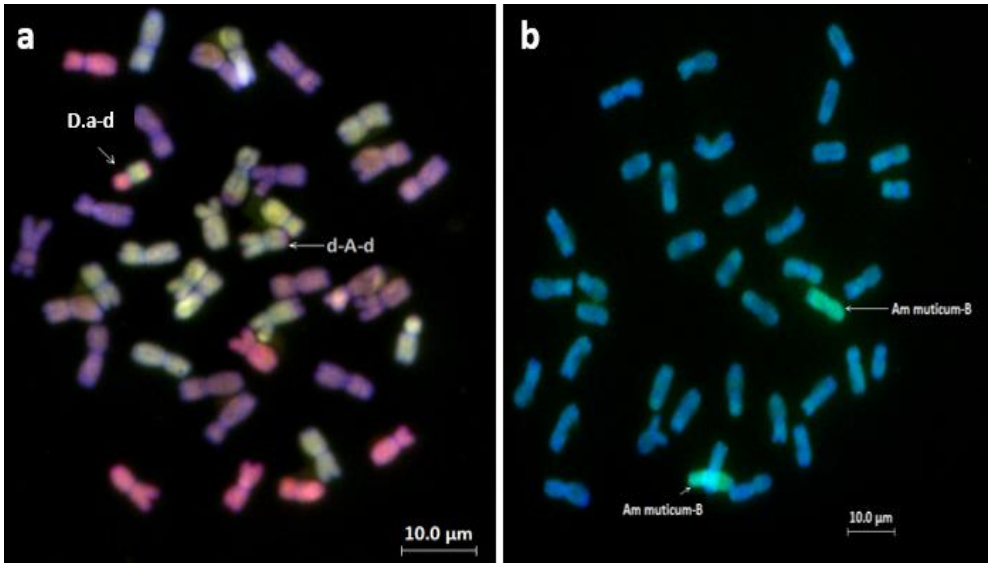

Figure 5-9. (a) Mc-GISH and (b) sc-GISH of root-tip metaphase spreads of BC_1-F_2-OR-25-B-B-B showing the presence of two D-genomic translocations (Chromosome colour-code: A: green, B: purple, D: red) and two T-genome (green) segments, respectively.

5.3.3.2 Mc-GISH analysis of the BC_1-F_2 lines in the crosses with "Karim"

Cytogenetic analysis of the self-progeny of the seven BC_1-F_1 lines that set seed in the cross combination with "Karim", showed that translocations were found only in the progenies of four BC_1-F_1 lines. A total of 20 BC_1-F_2 lines carrying genomic translocation involving the D-genome were identified (**Table 5-13**). In comparison to the previous generation, four new genomic translocations were identified in three of these lines (underlined translocation; **Table 5-13**). Three of the translocations involved the A with the B genome and only one involved the D-genome with the A-genome. With the exception of BC_1-F_2-KR-18-A-A-D that showed the presence of only two new D-a and A.b genomic translocations, the rest of the lines retained at least one copy of the

genomic translocation identified previously in the BC_1-F_1 parental lines (**Table 5-13**).

The BC_1-F_2 lines in the crosses with "Karim" showing translocations were all generated from WMI parental lines that had one or two *Am. muticum* segments and therefore these lines were also screened with sc-GISH for *Am. muticum* introgressions. Although the results weren't clear in four of the lines, 93% of the screened lines (15 out of 16 lines) had retained a single or homozygous T-genome segment translocated with a small B-genome segment in its telomeric long arm noted as a T-B translocation. The total chromosome counts of the different lines revealed that 70% of the 20 BC_1-F_2 lines were tetraploid (**Table 5-13**). Therefore, simultaneous introgression of the D- and the T-genomes was identified in ten of the tetraploid BC_1-F_2 lines. Two of these lines had a homozygous T-genome introgression substituting two B-genome chromosomes as well as a small D-genome segment translocated in the telomeric region of an A-genome chromosome long arm, in a total of 28 chromosomes. These two lines had a durum wheat head type and were fertile generating BC_1-F_3 seed. Another five fertile tetraploid lines showing a durum wheat head type, had single T-genome introgressions substituting a single B-genome chromosome alongside a single small D-genome segment introgression translocated in the telomeric region of either arms of an A-genome chromosome (e.g. **Figure 5-10**).

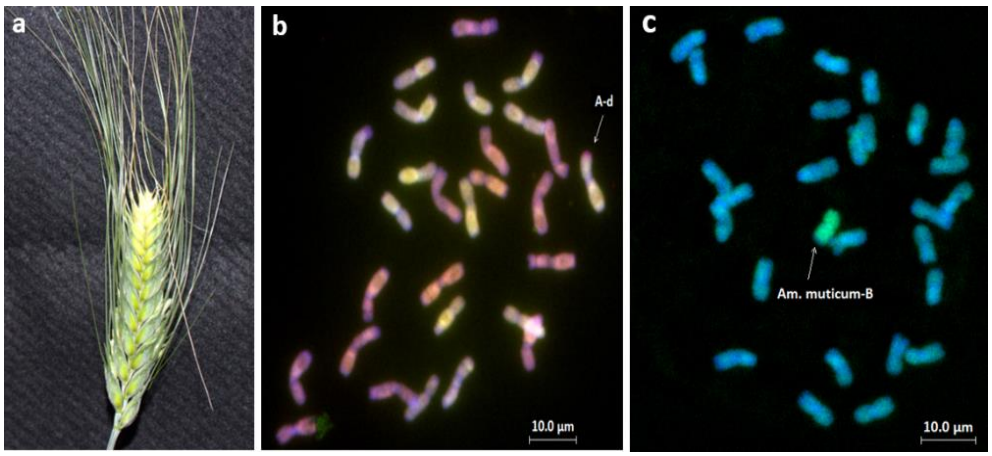

Figure 5-10. (a) Spike morphology, (b) mc-GISH and (c) sc-GISH of root-tip metaphase spreads of the tetraploid BC_1-F_2-KR-18-A-A-B showing (b) a single D-genome translocation (Chromosome colour-code: A: green, B: purple, D: red) and (c) a single T-genome (green) introgression.

Table 5-13. Selected BC_1-F_2 lines of "WMI lines/Karim" crosses carrying D-genomic translocations (Group 2).

Line No.	BC_1-F_2 lines reference	A-, B- and D-genome chrom. No.	Translocation type*No.	No. of T-genome segments	Total Chrom. No.	BC_1-F_3 seed produced
1	**BC_1-F_2-KR-18-A-A-A**	13A+12B+1D	A-d*1(LA)	2	29	200
2	**BC_1-F_2-KR-18-A-A-D**	12A+13B	D-a*1 + B-a*1	1	28	66
3	**BC_1-F_2-KR-18-A-A-B**	13A+12B	A-d*1(LA)	2	28	104
4	**BC_1-F_2-KR-18-A-C-A**	13A+13B	A-d*1(SA)	1	28	144
5	**BC_1-F_2-KR-18-A-C-D**	13A+13B	A-d*1(SA)	1	28	154
6	**BC_1-F_2-KR-18-A-C-E**	12A+14B	A-d*2(SA)	--	28	S
7	**BC_1-F_2-KR-18-A-C-F**	12A+14B	A-d*2(SA)	--	28	S
8	**BC_1-F_2-KR-18-A-C-G**	13A+14B	A-d*1(SA)	--	28	S
9	**BC_1-F_2-KR-18-A-D-A**	13A+13B	A-d*1 (LA)	1	28	106
10	**BC_1-F_2-KR-18-A-D-B**	13A+13B	A-d*1 (LA)	1	28	48
11	**BC_1-F_2-KR-18-A-D-D**	12A+12B	A-d*2 (LA) + B-a*1	1	28	S
12	**BC_1-F_2-KR-18-A-D-E**	12A+12B	A-d*2 (LA) + B-a*1	1	28	S
13	**BC_1-F_2-KR-18-A-D-I**	12A+12B	D.a-b*1 + A-d*2	2	29	75
14	**BC_1-F_2-KR-18-A-D-F**	12A+13B	D.a-b*1 + A.D*1+ A-d*1(LA)	2	30	142
15	**BC_1-F_2-KR-18-A-D-G**	13A+14B	A-d*1(LA)	--	28	19
16	**BC_1-F_2-KR-18-A-D-H**	13A+13B	A-d*1(LA)	1	28	1
17	**BC_1-F_2-KR-18-C-A-C**	9A+14B	D.a-b*1 + A.D*1+ A-d*2(LA)	2	29	N
18	**BC_1-F_2-KR-18-C-A-A**	13A+12B	A-d*1(LA)	2	28	51
19	**BC_1-F_2-KR-19-A-B-A**	14A+13B	d-A-d*1	1	29	S
20	**BC_1-F_2-KR-19-A-B-B**	14A+14B	d-A-d*1	0	29	70

NB: *No. indicates the number of copies, MS=Maturity stage, N=Plant didn't reach maturity stage; SA=translocation on the short arm; LA=translocation on the long arm; S=sterile; -- not identified, sister lines grouped together.

Tetraploid lines with more than one wheat-genomic translocation in the presence of a single T-genome introgression were identified. The tetraploid BC_1-F_2-KR-18-A-A-D line carrying single D-a and B-a translocations alongside a single T- segment introgression was fertile and had a durum wheat head type (**Table 5-13**). However, BC_1-F_2-KR-18-A-D-E and BC_1-F_2-KR-18-A-D-D tetraploid lines, both carrying three D-genome translocations (a homozygous A-d plus a single B-a translocation) in the presence of a single T-genome segment introgression, were sterile (**Table 5-13**). Lines with three and four D-genomic translocations and a homozygous T-B translocation were also identified (**Table 5-13**) although these lines mostly carried a total of 29 or 30 chromosomes. In fact, the line BC_1-F_2-KR-18-C-A-F carrying three D-genomic translocations (D.a-b, A.D and A-d) was found to be fertile, whereas, its sister line BC_1-F_2-KR-18-C-A-C carrying four genomic translocations was sterile.

5.4 Production and mc-GISH screening of the BC_1-F_3 generation

Approximately 83% of the BC_1-F_3 seed sown germinated with a total of 34 lines carrying D-genomic translocations identified (**Table 5-14**).

Table 5-14. Germination and screening of the BC_1-F_3 for D-genomic translocations.

BC_1-F_3 Cross-combination pedigree	No. of crosses selected	No. of Seed sown	No. of seed germinated	% of germination	No. of plants with translocation
WMI lines /Om Rabiaa 5*2	5	9	9	100	9
WMI lines /Karim*2	12	51	41	76.9%	26
Total	18	60	50	83.3%	**35**

5.4.1 Cytogenetic analysis of the BC_1-F_3 lines in the crosses with "Om Rabiaa 5"

5.4.1.1 Mc-GISH analysis

Homozygous introgressions of a small D-genome segment in the absence of *Am. muticum* segment were previously confirmed in five BC_1-F_2 sister lines of the crosses with "Om Rabiaa 5" (**Table 5-12**). Two randomly selected BC_1-F_3 from four of the lines were checked for the maintenance of the homozygous D-introgressions. Mc-GISH confirmed the presence of the D-introgressions in all

the lines. All the lines were homozygous for the A-d translocation and had a durum wheat head type (e.g. **Figure 5-11**). The screening of BC_1-F_3-224-A (the only self-progeny of BC_1-F_2-OR-25-B-B-B) revealed the retention of only the d-A-d translocation alongside a single T-B translocation and two univalent D-genome chromosomes in a total of 31 chromosomes (**Table 5-15**).

Table 5-15. Selected BC_1-F_3 lines of "WMI lines/Om Rabiaa 5" crosses carrying D-genomic translocations.

Grp.	Line No.	BC_1-F_3 line ref.	BC_1-F_2 Parental line ref.	A-, B- and D-genome chrom. No.	Transloc-ation type$^{*No.}$	No. of T-genome segments	Total Chrom. No.
G1	1	BC_1-F_3-500-A	BC_1-F_2-OR-7-B-A-A	13A+14B	A-d^{*2}	0	28
	2	BC_1-F_3-500-B		13A+14B	A-d^{*2}	0	28
	3	BC_1-F_3-501-A	BC_1-F_2-OR-7-B-A-C	12A+14B	A-d^{*2}	0	28
	4	BC_1-F_3-501-B		12A+14B	A-d^{*2}	0	28
	5	BC_1-F_3-502-A	BC_1-F_2-OR-7-B-A-D	12A+14B	A-d^{*2}	0	28
	6	BC_1-F_3-502-B		12A+14B	A-d^{*2}	0	28
	7	BC_1-F_3-237-A	BC_1-F_2-OR-7-B-A-E	12A+14B	A-d^{*2}	0	28
	8	BC_1-F_3-237-B		12A+14B	A-d^{*2}	0	28
G2	9	BC_1-F_3-224-A	BC_1-F_2-OR-25-B-B-B	14A+15B+ 2D	d-A-d^{*1}	1	31

NB: $*^{No.}$ indicates the number of copies, sister lines grouped together, G1= WMI parental lines without T-genome segment, G2= WMI parental lines carrying T-genome segments.

Figure 5-11. (a) Plant and (b) spike morphology of the tetraploid BC_1-F_3-500-B line.

5.4.1.2 Mc-FISH analysis

MC-FISH analysis of BC_1-F_3 lines carrying the homozygous A-d (SA) translocations, confirmed the presence of full sets of the A and the B chromosomes. The telomeric D-genome segment introgressed into the A-genome (e.g. BC_1-F_3-501-A) is very small in size, and thus too difficult to precisely characterize the D-genome segment. When compared to the mc-FISH karyotype of Chinese Spring, one possibility is that the small D-genome segment could be the telomeric region of the 3DS chromosome arm translocated with the short arm of chromosome 5A. However, further confirmation with molecular markers should be considered (e.g. **Figure 5-12**).

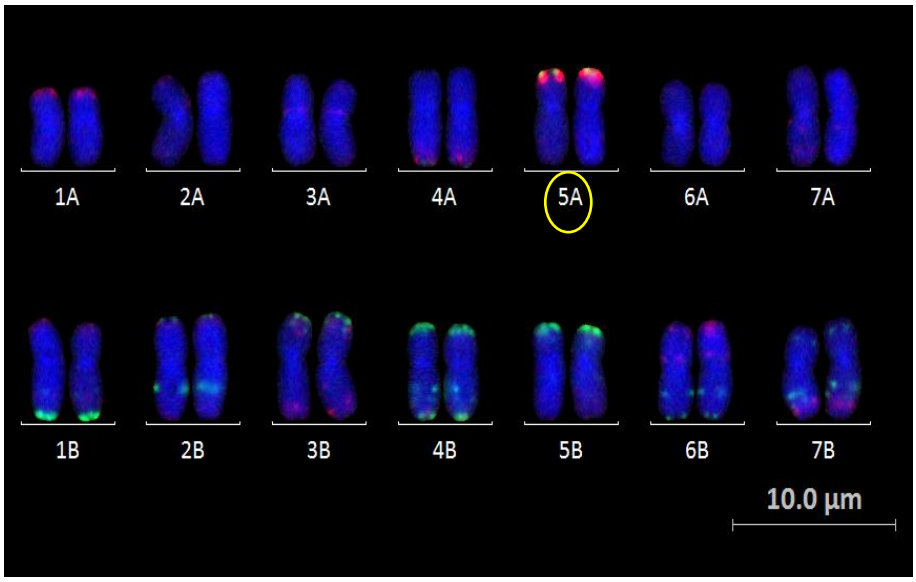

Figure 5-12. FISH karyotype of BC_1-F_3-501-A using the Oligo-pAs.1 (red) and Oligo-pSc119.2 (green) probes counterstained with DAPI (blue) showing a D-introgression into the 5A chromosomes (yellow circle).

5.4.2 Cytogenetic analysis of the BC_1-F_3 lines in the crosses with "Karim"

5.4.2.1 Mc-GISH analysis

D-genomic translocations were shown to be present in the self-progeny of only ten of the twelve fertile BC_1-F_2 lines. When compared to the translocations identified in the parental lines, all the lines retained one or two copies of the D-translocation previously identified (**Table 5-16**). Considering the total chromosome number, 88.4% of the screened BC_1-F_3 lines carrying D-genomic translocation were tetraploid. Homozygous D-genome introgressions of the "A-d (SA)" translocation type were identified in 52% of these lines (12 out of 23 lines) originating from a single D-translocation in the parental line. In addition, a single copy of the same A-d translocation type was identified in 43% of the BC_1-F_3 tetraploid lines (10 lines out of 23 lines). Adding the labelled genomic DNA probe of *Am. muticum* to the wheat mc-GISH allowed the simultaneous screening for the D-genomic translocations and the retention of the T-genome segments. Results showed that all of BC_1-F_3 lines had retained at least one T-genome segment. This T-genome segment was translocated with a small B-genome segment in its telomeric long arm region noted as a T-B translocation.

Table 5-16. Selected BC_1-F_3 lines of "WMI lines/Karim" crosses carrying D-genomic translocations.

Lines No.	BC_1-F_3 line reference	BC_1-F_2 parental line ref.	A-, B- and D-genome chrom. No.	Translocation type$^{*No.}$	T-genome segment	Total Chrom. No.
1	BC_1-F_3-141-A	BC_1-F_2-KR-18-A-C-A	13A+12B	A-d^{*1} (SA)	2	28
2	BC_1-F_3-141-C		13A+12B	A-d^{*1} (SA)	2	28
3	BC_1-F_3-141-D		13A+12B	A-d^{*1} (SA)	2	28
4	BC_1-F_3-144-B	BC_1-F_2-KR-18-A-C-D	13A+12B	A-d^{*1} (SA)	2	28
5	BC_1-F_3-202-A	BC_1-F_2-KR-18-A-A-A	12A+12B	A-d^{*2} (LA)	2	28
6	BC_1-F_3-202-C		12A+12B	A-d^{*2} (LA)	2	28
7	BC_1-F_3-202-D		12A+12B	A-d^{*2} (LA)	2	28
8	BC_1-F_3-214-B	BC_1-F_2-KR-18-A-D-D	12A+13B	A-d^{*2} (LA)	1	28
9	BC_1-F_3-214-C		13A+12B	A-d^{*1} (LA)	2	28
10	BC_1-F_3-215-B	BC_1-F_2-KR-18-A-D-D	13A+13B	A-d^{*1} (LA)	1	28
11	BC_1-F_3-217-B	BC_1-F_2-KR-18-A-A-D	13A+12B	D-a^{*1} (LA)	2	28
12	BC_1-F_3-217-C		13A+12B	A-d^{*1} (LA)	2	28
13	BC_1-F_3-314-A	BC_1-F_2-KR-18-A-D-H	13B+12B	A-d^{*1} (LA) + <u>B-A-d^{*1}</u>	1	28
14	BC_1-F_3-312-A	BC_1-F_2-KR-18-A-D-F	12A+12B	A-d^{*2} (LA)	2	28
15	BC_1-F_3-312-B		13A+12B	A-D^{*1} + D.a-b^{*1}	2	29
16	BC_1-F_3-312-D		12A+12B	A-d^{*2} (LA)	2	28
17	BC_1-F_3-312-E		12A+12B	A-d^{*2} (LA)	2	28
18	BC_1-F_3-312-F		13A+12B	A-d^{*1} (LA)	2	28
19	BC_1-F_3-315-A	BC_1-F_2-KR-18-A-D-I	12A+12B	A-d^{*2} (LA)	2	28
20	BC_1-F_3-315-B		12A+12B	A-d^{*2} (LA)	2	28
21	BC_1-F_3-315-C		12A+12B	A-d^{*2} (LA)	2	28
22	BC_1-F_3-315-D		11A+12B	A-d^{*1} (LA) + D.a-b^{*1}	2	27
23	BC_1-F_3-315-E		12A+12B	A-d^{*2} (LA)	2	28
24	BC_1-F_3-315-F		12A+12B	A-d^{*2} (LA) + D.a-b^{*1}	2	29
25	BC_1-F_3-324-B	BC_1-F_2-KR-18-C-A-A	10A+12B	A-d^{*2} (LA)	4	28
26	BC_1-F_3-324-C		11A+12B	A-d^{*1} (LA)	4	28

NB: *$^{No.}$ indicates the number of copies, sister lines grouped together.

In all lines, the single or homozygous T-B translocation substituted a single or two B-genome chromosomes. Therefore, simultaneous introgression of the T-genome and the D-genome was seen in all of the BC_1-F_3 tetraploid lines. All these lines successfully reached maturity and were fertile.

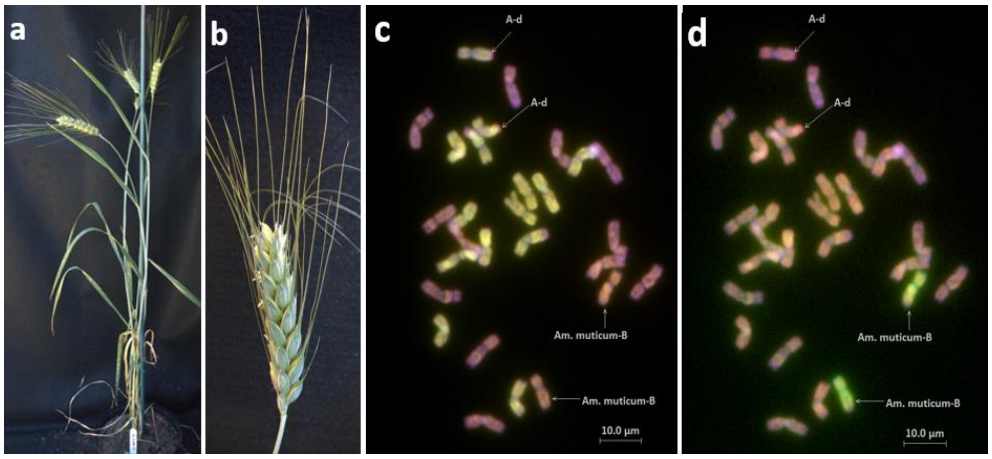

Figure 5-13. (a) Plant, (b) spike morphology, (c, d) mc-GISH of the same root-tip metaphase spread of the tetraploid BC_1-F_3-202-C showing simultaneous (c) homozygous D-genome translocations (Chromosome colour-code: A: green, B: purple, D: red) and (d) T-genome (green contrast) introgressions.

Only one new B-A-d translocation was identified. This was present in BC_1-F_3-314-A, alongside the retained A-d translocation and one T-genome segment in a total of 28 chromosomes (**Table 5-16**). However, this line was sterile and failed to set seed. Two selfed-lines retained four T-genome segments (the parental line had two T-B translocations). The genomic composition of these two lines showed that the four T-B translocations substituted two A- and two B-genome chromosomes, and also present was either a single or homozygous small D-genome segment introgressed into an A-genome chromosome long arm. These two lines failed to reach maturity.

5.4.2.2 Mc-FISH analysis

Mc-FISH analysis of the "A-d (SA)" translocation (**Table 5-16**, lines1-4) revealed that the small introgressed D-genome segment was most likely translocated with chromosome 2A in the telomeric region of the short arm. The FISH karyotype analysis of these lines also showed that the large homozygous T- segments substituted the pair of 4B chromosomes in a total of 28 chromosomes (e.g. **Figure 5-14**). Mc-FISH analysis of the "A-d (LA)" translocation, retained as either a homozygous or a single translocation in 16 BC_1-F_3 lines (**Table 5-16**: lines 8-24) showed that the small D-genome segment

introgressed was most likely translocated in the telomeric region of the 7A chromosome long arm (e.g. **Figure 5-15**). Again, the single or homozygous T-B translocation substituted either a single or both copies of chromosome 4B, respectively. However, the D-genome segment as well as the B-genome segment translocated with the T-genome, were too small in size and couldn't be characterised. Mc-FISH analysis also enabled the characterisation of the D.a-b translocation as 2DL.4AL. The 4AL chromosome of wheat is characterised by the presence of a conserved 7BS translocation in its telomeric region (Devos *et al.*, 1995) and hence the visualisation of these translocations as D.a-b using mc-GISH. The FISH karyotype analysis of this line revealed the presence of a 2DL.4AL-7BS translocation in addition to the homozygous 7A-d and T-B translocations in a total of 29 chromosomes (e.g. **Figure 5-16**).

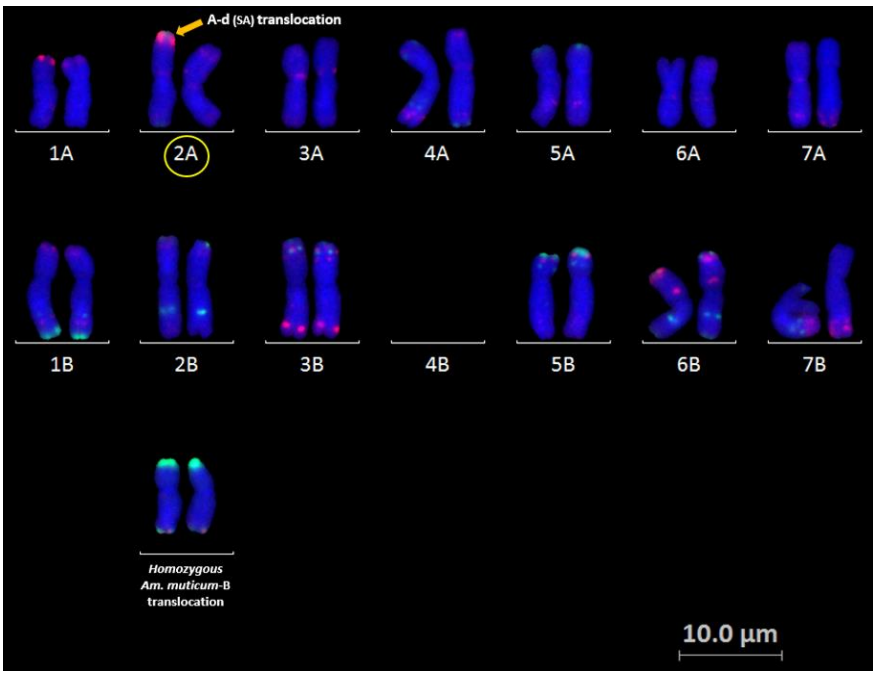

Figure 5-14. FISH karyotype of BC_1-F_3-141-C using the Oligo-pAs.1 (red) and Oligo-pSc119.2 (green) probes counterstained with DAPI (blue) showing a single D-genome introgression into the 2A chromosome (yellow circle).

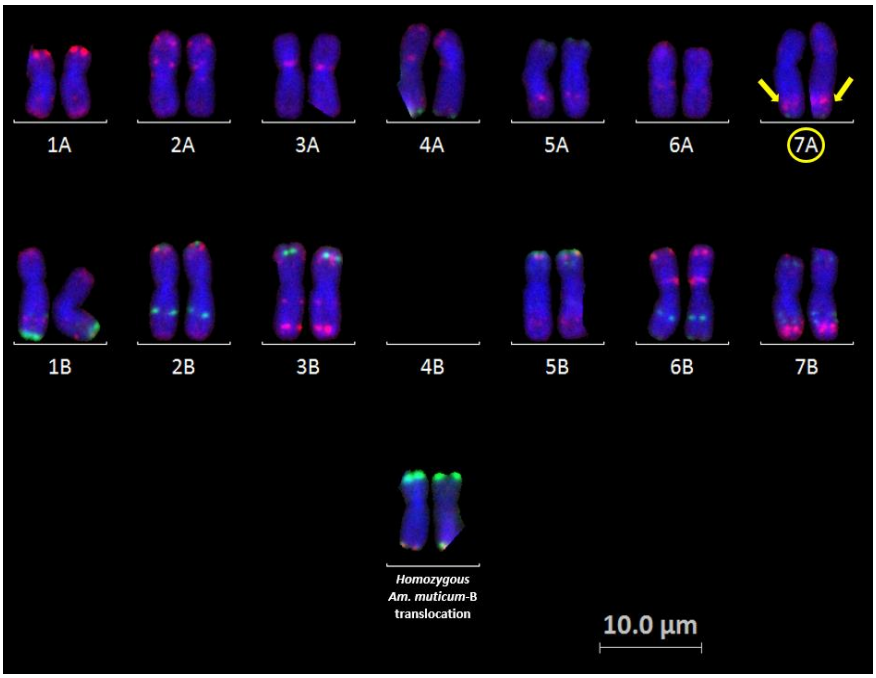

Figure 5-15. FISH karyotype of the tetraploid BC_1-F_3-315-E line using the Oligo-pAs.1 (red) and Oligo-pSc119.2 (green) probes counterstained with DAPI (blue) showing homozygous D-genome introgressions in chromosome 7AL (yellow arrows) and a homozygous T-B/4B substitution.

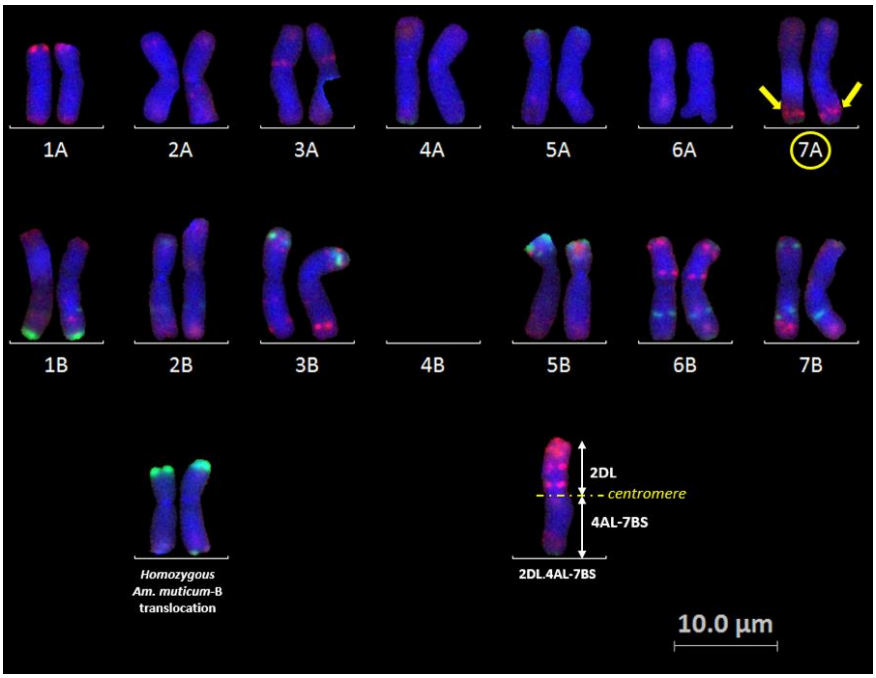

Figure 5-16. FISH karyotype of BC_1-F_3-315-F using the Oligo-pAs.1 (red) and Oligo-pSc119.2 (green) probes counterstained with DAPI (blue) showing homozygous D-genome introgressions in chromosome arm 7AL (yellow arrows), a 2DL.4AL chromosome translocation and a homozygous T-B translocation.

5.5 Discussion

Pentaploid crosses between bread and durum wheat are known to generate viable F_1 seed that can be used to backcross to either of the parents depending on the crossing aim. The presence of genomic rearrangements in the hexaploid background of alien/wheat introgression lines can form an alternative crossing strategy for D-genome introgression into durum wheat through these pentaploid crosses.

Genomic rearrangements involving the D-genome with either the A and/or the B-genome were distinguished in the advanced BC_1-F_3 and BC_4-F_1 generation of a wheat/Am. *muticum* introgression (WMI) crossing program (King *et al.,* 2017). While the overall aim was to introgress the D-genome segments into durum, these crosses also have the potential to increase the genetic variability of the A- and the B-genomes through recombination with their homologues of bread wheat. For the successful establishment of a viable pentaploid F_1 hybrid, the higher ploidy level genotype is usually used as the maternal parent (Padmanaban *et al.,* 2017a; b). Thus, using the hexaploid parent as the female parent in the pentaploid crosses is generally more successful in producing viable progeny (Ramsey and Schemske, 1998; Kalous *et al.,* 2015). Therefore, in our study the hexaploid WMI lines were used as the female parent and viable F_1 seeds were obtained from both of the cross-combinations with the two *T. durum* genotypes.

A higher number of F_1 seed was produced per cross compared to the number of BC_1 seed with either "Om Rabiaa 5" or "Karim" (**Figure 5-5**). The imbalanced chromosome number in the gamete of the pentaploid F_1 individuals, which in this study varied from 33 to 35 chromosomes, can impact pollen development and subsequent fertilization (Kihara, 1982). The affected pollen grains do not germinate, the pollen tubes fail to reach the ovary, or the male and female gametes fail to fuse (Sharma and Gill, 1983), all of which can explain the low number of BC_1 seed produced from the F_1 hybrids. For instance, pentaploid hybrids derived from a cross between *T. timopheevii* (AAGG) and *T. aestivum* produced completely sterile white anthers with infertile pollen (Bhagyalakshmi *et al.,* 2008). A higher seed set was obtained in the F_1 and the BC_1 generations using the "Om Rabiaa 5" genotype compared to "Karim". (**Figure 5-5**). Hence, the choice of parental genotype plays a key role in the

production of viable pentaploid hybrids (Padmanaban *et al.*, 2017a). Although the crosses with both of the durum wheat genotypes set viable pentaploid F_1 seeds, the results suggest a higher crossing compatibility using the "Om Rabiaa 5" genotype in the crosses with bread wheat compared to "Karim".

The majority of existing genotyping systems are based on dominant markers, which fail to differentiate between the presence of either a single or homozygous copy of a particular locus. In such case, it's impossible to detect incomplete or partial chromosomes in the presence of their complete homologous chromosome. Hence, cytological techniques, such as GISH or FISH can provide a more systematic approach for analysing chromosomal translocations (Eberhard *et al.*, 2010).

In this study, cytological approaches were used to characterise the genomic composition of the lines produced and to screen for the presence of the D-genome rearrangements. The mc-GISH technique has the ability to distinguish between the three genomes of wheat and therefore to visualise genomic rearrangements between the wheat sub-genomes. This technique has been widely used for studying intergenomic translocations and alien introgressions, and for discriminating between different genomes in polyploid cereals (Schwarzacher *et al.*, 1989; 1992; Schubert *et al.*, 2001; Silva and Souza, 2013).

As expected, after backcrossing the lines to the durum wheat parent, the average chromosome number gradually decreased due to the gradual loss of the univalent D-genome chromosomes. This reduction of chromosome number down to 28 chromosomes, was positively correlated with the improvement of the percentage of germination in both the cross-combinations, except for the BC_1-F_3 generation in the cross-combination with "Karim" (**Figure 5-18**).

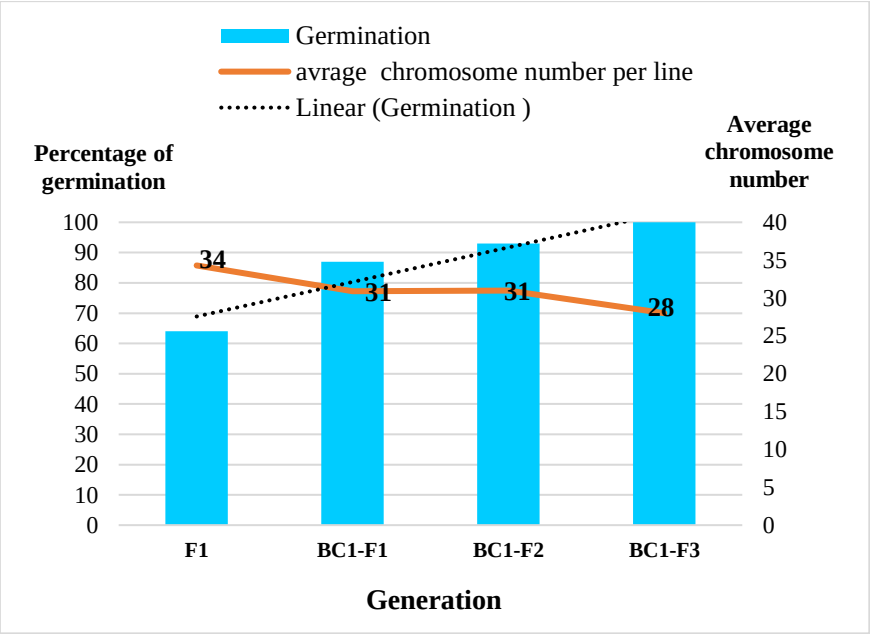

Figure 5-17. Percentage of germination (histogram) and the average chromosome number per line (curve) of the lines carrying D-genome translocations in the cross-combinations with "Om Rabiaa 5" at the F_1, the BC_1-F_1 and the BC_1-F_2 generations.

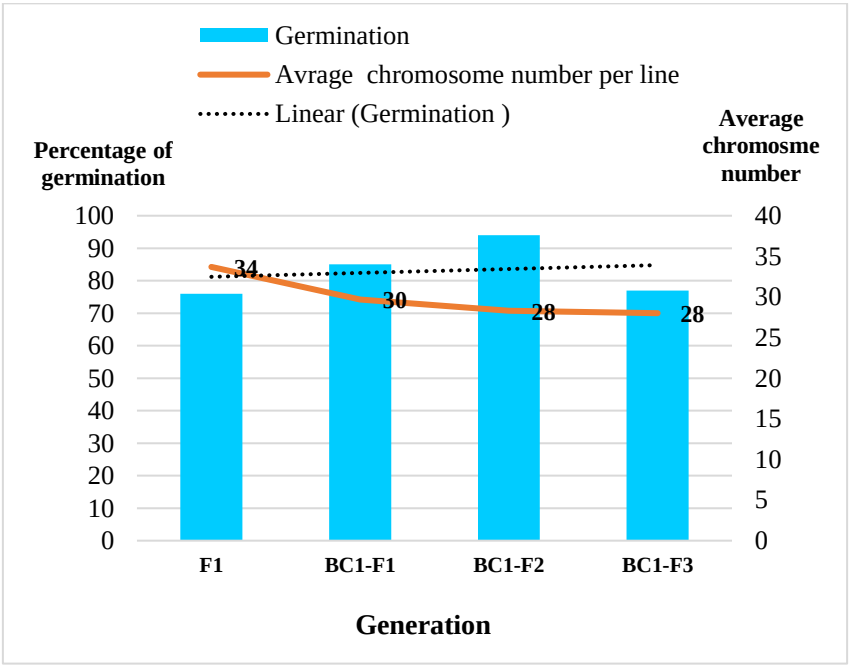

Figure 5-18. Percentage of germination (histogram) and the average chromosome number per line (curve) of the lines carrying D-genome translocations in the cross-combinations with "Karim" at the F_1, the BC_1-F_1 and the BC_1-F_2 generations.

A higher number of lines carrying D-genomic translocations was distinguished in the progenies of the durum wheat parent "Karim" in all generations compared to "Om Rabiaa 5" (**Figure 5-19**). This could indicate a higher tolerance of the "Karim" genotype for the presence of the genomic

translocations. Additionally, new genomic translocations involving the D- with either the A- and/or the B-genome, or the A- with the B-genome were identified in the different generations from the F_1 to the BC_1-F_2. Moreover, at all generations from the F_1 to the BC_1-F_3, lines classified in the G1 group had retained only one D-genome translocation, whereas several lines belonging to the G2 group showed the presence of more than one D-genome translocation type.

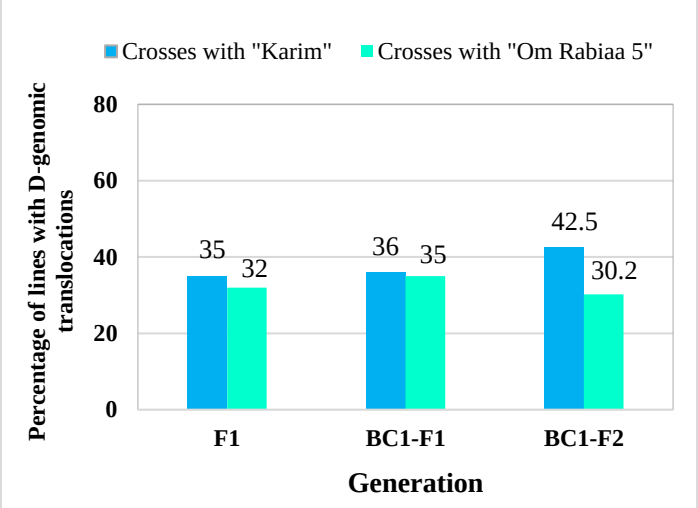

Figure 5-19. Percentage of lines shown D-genomic translocations in the two cross-combinations with either "Karim" or "Om Rabiaa 5" genotypes in the F_1, the BC_1-F_1 and the BC_1-F_2 generations.

A closer look at the number of lines carrying D-genome rearrangements, showed an equal or higher number of lines identified in the G2 group compared to the G1 group (**Figure 5-20** and **Figure 5-21**). In addition, new genomic translocations appeared to be occurring only in the lines belonging to the G2 group. This could be explained by the presence of four genomes together (A, B, D and T) in one cell with an unequal chromosome number that can promote an abnormal meiotic behaviour leading to homoeologous paring and therefore new genomic translocations. It has been shown in previous studies that combining two or more different genomes into one cell may cause changes in chromosome morphology, including differences in the size, thickening, or lengthening of chromosomes, a phenomenon referred to as genome shock, in addition to chromosomal rearrangements followed by a potential gain or loss of chromosomal segments (reviewed in Matsuoka, 2011). Moreover, *Am. muticum* species is known to harbour gene that promotes pairing between homoeologous

and suppresses the effect of the *Ph1* gene in hybrids with allopolyploid wheat (Dvorak, 1972; Dover and Riley, 1972).

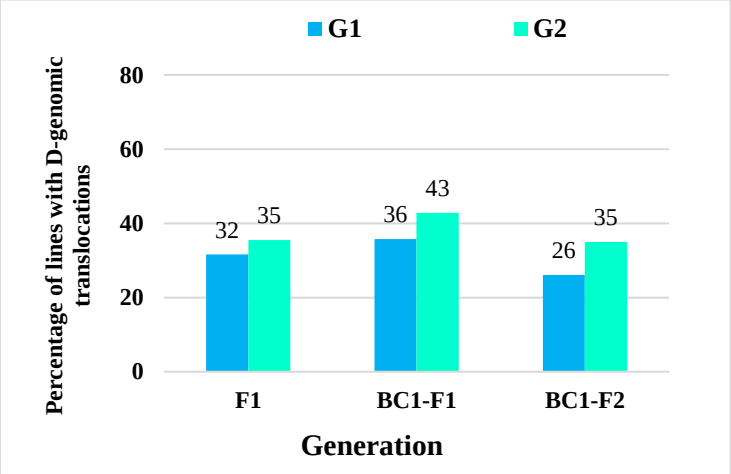

Figure 5-20. Percentage of lines showing D-genome translocations in the G1 and G2 groups of the crosses with "Om Rabiaa 5" at the F_1, BC_1-F_1 and BC_1-F_2 generations.

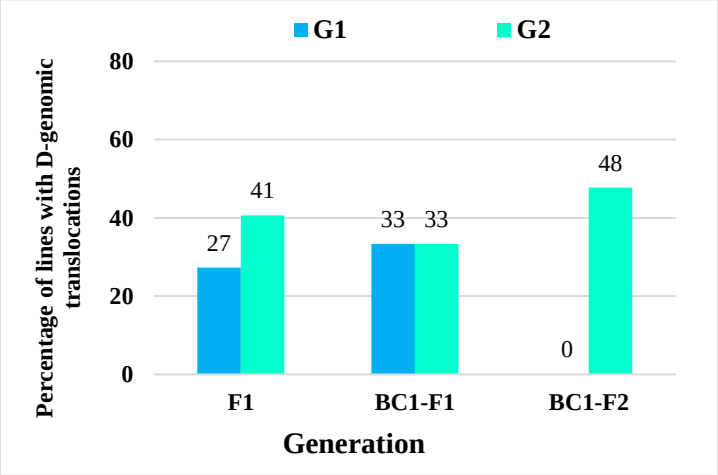

Figure 5-21. Percentage of lines showing D-genome translocations in the G1 and G2 groups of the crosses with "Karim" at the F_1, BC_1-F_1 and BC_1-F_2 generations.

A higher number of different AD (e.g. A-d, d-A-d, D-a and A.D) and ABD (e.g. D.a-b, D-a-b, D.a-d and B.a-d) translocation types were identified compared to the BD types (e.g. B.D and B-d) (**Figure 5-22**). A few lines with AB translocations were also identified (e.g. A.B and B-a). The A-genome assembly analysis of bread wheat and five diploid related species supports that both at the base-pair level and in gene content, the A- and B-genome lineages are more similar to the D-genome lineage than they are to each other (Marcussen *et al*., 2014). Hence, pairing between the A- and the D-genomes consistently observed in wheat hybrids denotes a much lower differentiation between these

two genomes than between the A- and B- or B- and D-genomes, at least in the regions of high recombination in the distal chromosome regions (Marcussen *et al.*, 2014). This is consistent with the high level of AD translocations types observed in the present study, especially in the telomeric regions of the chromosomes. This result suggests a higher association rate between the A- and the D-genomes takes place in the wheat/ *Am. muticum* derivative introgression lines.

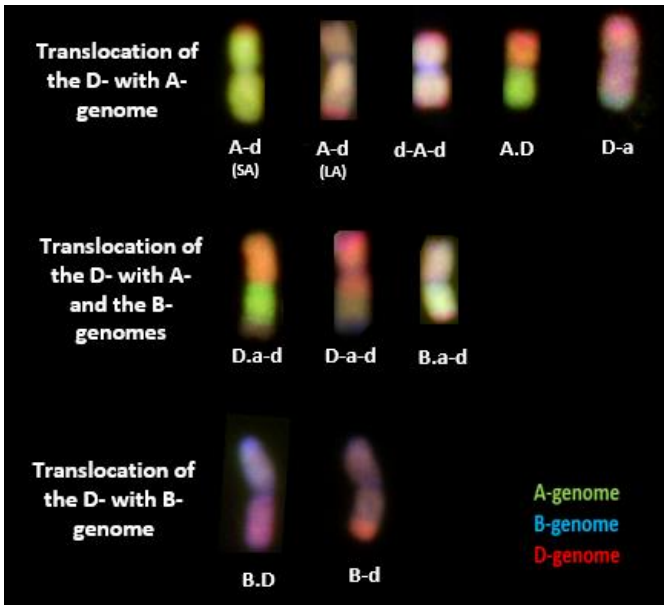

Figure 5-22. Mc-GISH of the different AD, ABD and BD translocation types identified.

Independently of the total chromosome number, mc-GISH screening results of the BC_1-F_2 and BC_1-F_3 generations showed no clear positive or negative correlation between the number of the different D-genomic translocation types present per line and its viability/fertility in both of the cross-combinations. For instance, in the BC_1-F_2 generation of the crosses with "Karim" some of the tetraploid lines with a single D- segment introgression were sterile (e.g. **Table 3-2**, lines 6-8), whereas other lines with three different genome translocations involving the D-genome were fertile and set BC_1-F_3 seeds (e.g. **Table 3-2**, line 14). Opposite cases were also found, where lines with a single or homozygous D-introgression were fertile, while lines with two or more different D-translocation types were either sterile or failed to reach maturity (e.g. **Table 3-2**; Lines 11 and 17). The screening of these lines for the presence of the T-genome revealed that some of these lines had one or two large T-genome segments

translocated with a small B-genome segment. Thus, the failure to reach maturity, or the sterile phenotype, might be related to the simultaneous presence of the four A, B, D and T-genomes in the same cell with an unbalanced chromosome number. However, the presence of different D- and/or T- genome segments or the loss of A- or B-genome segments leading to either the activation and/or suppression or the loss of some genes, respectively, directly affecting plant growth or fertility also need s to be considered.

Of the different D-translocations described in the WMI lines, only telomeric small D-genome segments were successfully introgressed into the tetraploid background of both of the durum wheat varieties used, through a telomeric translocation in either arm of an A-genome chromosome. These results indicate that translocations of smaller size have a higher chance to be translocated compared to the larger translocated D-genome segments. Furthermore, as expected when the translocated chromosomes were present as additions, they are mainly lost. Monosomic additions are less stable than substitutions at meiosis due to the lack of pairing. Whereas, when the translocated chromosomes substitute one of the wheat chromosomes, they had a higher chance to be retained and introgressed.

Tetraploid lines with single or homozygous D-genome introgressions were identified in the BC_1-F_3 generation in both of the cross-combinations. However, when comparing consecutive generations for the retention of the D-genome translocations, mc-GISH analysis showed that none of the translocations previously identified in the parental G1-WMI lines, were retained in the progeny of the cross-combination with “Karim”. However, both newly formed or previously identified D-genome translocations in the G2-WMI lines were introgressed into the tetraploid background of “Karim”. All of the single or homozygous D-genome introgressions in the tetraploid BC_1-F_3 lines in the background of “Om Rabiaa 5” were previously identified in the parental WMI lines belonging to the G1 group. In comparison, the introgressed D-genome segments in the background of “Karim”, were newly formed in the BC_1-F_1 generation in group G2 and retained as a single or homozygous introgression in the BC_1-F_3 generation.

Sc- or mc-GISH screening, revealed that all of the 26 BC_1-F_3 lines from the crosses with “Karim” had retained at least one large T-genome segment

translocated with a telomeric B-genome segment substituting a B-genome chromosome. Therefore, simultaneous introgressions of the D- and the T-genomes were identified in the BC_1-F_3 lines. This simultaneous introgression of the D-and the T-genomes in the tetraploid background of "Karim" can explain the reduction-in the germination rate of the progeny from Karim observed in the BC_1-F_3 generation (**Figure 5-18**). In particular, nine of these lines were tetraploid with a simultaneous homozygous introgressions of the D- and T-genomes. These lines were fertile and had a durum wheat head type. However, only one line carrying a single T-genome segment was identified in the BC_1-F_3 lines of the cross-combination with "Om Rabiaa 5". These results suggest the lower tolerance of the "Om Rabiaa 5" genotype for the presence of the T-genome segments compared to the "Karim". Hence, the choice of the durum wheat genotype plays a major role for the successful introgression of the D-genome and/or the alien species in durum wheat.

Mc-FISH is a labour-intensive, cytogenetic technique, requiring a high level of operator expertise, which does not lend itself to high throughput screening of population lines. Nevertheless, this cytological technique provides the possibility, not only to distinguish between the three different sub-genomes of wheat, but also to allocate every chromosome to its appropriate genomic group according to a karyotype of Chinese Spring developed by Tang *et al.* (2014). The presence of the 5B-chromosome pair supports the conclusion that the newly formed wheat sub-genome translocations weren't the result of the lack of the *Ph1* gene, but could more probably be accounted for by the presence of four different genomes in one cell as described above or the potential presence of a *Ph1* suppressor gene on the T-genome segment retained.

Additionally, mc-FISH analysis of the D.a-b translocation allowed the characterisation of this particular translocation as 2D.4AL. This line had this particular translocation as an addition as well as a homozygous telomeric D-translocation in the long arm of chromosome 7A and a homozygous T-B translocation substituting the pair of 4B chromosomes in a total of 29 chromosomes. The 4AL chromosome arm of wheat harbours a conserved telomeric translocation with the telomeric region of the 7BS chromosome arm and was part of a cyclic translocation involving chromosomes 4A/5A/7B. This translocation occurred during wheat evolution at the tetraploid level, i.e. before

the second polyploidization event which formed hexaploid wheat (Devos *et al.*, 1995).

All the BC_1-F_3 lines with either a single or a homozygous T/4B substitution appeared to be have a normal growth cycle and were completely fertile. Thus, the 4B chromosomes nullisomy did not affect the fertility in these lines. In fact the Langdon durum 4D (4B) disomic substitution line is fertile and can be selfed in the absence of the 4B chromosomes (Joppa and William, 1988). This therefore suggests that the introgressed T-genome segment fully compensates for the absence of the 4B chromosomes but doesn't contain a gene(s) that affects the fertility in these lines.

Taken in consideration the results of the chapter 3, were some of the Langdon D-genome disomic substitution lines were seen to have a differential reaction to STB disease depending on the presence/absence of certain chromosome pairs, the lines developed in the present chapter can be screened to STB disease. The comparison of both results can potentially either confirm or deny the hypothesis that the D-genome of bread wheat can be used as a source of resistance to *Z. tritici* in durum wheat.

To our knowledge, no previous investigation has been conducted to purposely introgress D-genomic rearrangements, present in hexaploid wheat/alien introgression lines, into durum wheat. This might be due to not only the focus of researchers on the alien chromatin introgression, but also to the limited access or use of the GISH technique in the past. Nerveless, nowadays with the advances in cytological techniques and the possibility of a mc-GISH approach to visualise different genomes simultaneously, it has become easier to identify these translocations in the background of the wheat/alien introgression lines (King *et al.*, 2017).

Conclusion:

The present study, the hypothesis of the transfer of wheat sub-genomic rearrangements, as well as alien segments from hexaploid derived wheat/alien introgression lines into durum wheat through pentaploid crosses, was demonstrated to be a successful crossing strategy. The choice of the parental durum genotype played a major role in the direction of the cross. For instance, according to the data presented here, the durum wheat "Karim" appears to be more receptive/tolerant to the presence of wheat genomic rearrangements and

Am. muticum introgressions than “Om Rabiaa 5”. This crossing strategy was shown to be an efficient crossing approach that can be used to introgress D-genome segments into durum wheat. Once the D-genome segments become homozygous in the background of the tetraploid durum, KASP molecular markers will be used to characterise the introgressed D-genome segment.

6 Chapter VI. General discussion

STB, caused by *Z. tritici*, is currently the most serious foliar disease of wheat grown under temperate (15–20°C) and humid climates in Europe, South America, North Africa, and Central Asia.

Genetic variability combined from hexaploid and tetraploid wheat into a pentaploid hybrid has great potential for crop improvement (Eberhard *et al.,* 2010; Martin *et al.,* 2013; Kalous *et al.,* 2015). Therefore, one of the objectives of the present thesis was the use of pentaploid crosses through interspecific hybridisation between three resistant hexaploid wheats (KK4500, SH M3 and Synthetic 6X) and two susceptible tetraploid wheat (Karim and Om Rabiaa5) in an attempt to improve the level of Septoria resistance in durum wheat.

The durum wheat cultivars used in these crosses are known to have good agronomic traits. “Karim” has been cultivated in Tunisia since 1973 and was registered in the official Tunisian catalogue in 1982. “Om Rabiaa 5” was released in 1981 by ICARDA and has been cultivated in Tunisia since 1987 and was proven to be an excellent parent with several recent releases derived from its hybridization in the ICARDA durum wheat breeding program (Nachit *et al.,* 2016). More than 20 years after its release, “Karim” continues to be one of the most sown durum wheat variety in Tunisia. Likewise, “Om Rabiaa 5” which was selected for its good drought tolerance in the semi-arid region is still cultivated today in 21 countries, mostly in the driest environments by smallholder farmers (Latican *et al.,* 2016). Recent study by Slama et *al*. (2018) showed that both of the cultivars still harbour a better tolerance to drought stress with a stable yield in Tunisia, comparing to the modern durum wheat varieties. However, these two cultivars are highly susceptible to STB disease in the northern part of Tunisia, where most of the wheat growers are localised. Hence, the choice of using “Karim” and “Om Rabiaa 5” in the present study, to

improve their resistance to STB disease and combine it with the good agronomic traits present in these two durum wheat varieties.

Two crossing strategies were undertaken. The first targeted the transfer of the *Stb* genes present on the A-genome and consisted of a direct cross between the resistant hexaploid and the susceptible tetraploid followed by backcrossing of the F_1 pentaploid to the durum parent. Preliminary screening of the durum wheat cultivar "Karim" and Om Rabiaa" for the presence of the *Stb6* (3AS), *Stb7* (4AL), *Stb12* (4AL) and *Stb17* (5AL) present on the A-genome undertaken in this study, revealed the presence of the two closely linked genes *Stb7* and *Stb12*. Their presence in the susceptible genomic background of these two cultivars indicates that they are either ineffective against the Tunisian *Z. tritici* populations or their expression is suppressed by suppressor gene(s) in durum wheat. Field based phenotyping also revealed the ineffectiveness of *Stb6*, and *Stb17* when introgressed into the genomic background of either "Karim" or "Om Rabiaa 5". This is in contrast to what has been previously demonstrated for some other wheat diseases such as yellow rust (Xu *et al.,* 2013) and powdery mildew (Rong *et al.,* 2000), where the resistance genes *Yr53* and *Pm26* respectively, conferred a good level of resistance in durum wheat when transferred from bread wheat using pentaploid crosses. The results of the present study demonstrate that at least for *Stb6 and Stb17,* genes present on the A-genome of hexaploid wheat cannot be used for durum wheat improvement to STB disease. Unlike the examples above for yellow rust and powdery mildew, this was also seen for a major locus on 3BL conferring a high level of resistance to *Fusarium* crown rot (FCR) in hexaploid wheat failed to provide any improvement in resistance in durum wheat (Ma *et al.,* 2012). Hence, resistance loci of the hexaploid wheat may not be functional in a durum wheat background for some diseases.

To facilitate the retention/transfer of the *Stb* genes present on the D-genome into the tetraploid background, a second crossing strategy was followed using the LND-DS lines caring the pair of the D-genome of the same group as where the *Stb* gene is located in the bread wheat parent. The F_1 pentaploid hybrids produced were then top and backcrossed to both of the susceptible durum wheat parent. Unfortunately, *Stb* 5 nor *Stb10* were transferred into the genomic background of "Karim". Thus, screening and tracing of these genes via

molecular markers and cytogenetic analysis in the early generations should be considered for more efficient introgression.

With five of the known *Stb* genes mapped to the D-genome of either the bread wheat (Adhikari *et al.,* 2004a; Chartrain *et al.,* 2005a; Ghaffary *et al.,* 2011) or *Ae. tauschii* through the use SHW (Arraiano *et al.,* 2001b; Ghaffary *et al.,* 2012), the effect of the D-genome on STB disease resistance in durum wheat were assessed through the phenotyping of the full set of LND-DS lines (Joppa and Williams, 1988) at both the seedling and adult stages under controlled and field conditions, respectively. Results revealed a differential reaction between the two growth stages. It has previously been reported that for STB disease, the resistance genes do not necessarily provide resistance during all growing stages. Some of the *Stb* genes were reported to provide resistance only at the seedling stage, e.g. *Stb9* (Chartrain *et al.,* 2009), whereas *Stb17* was expressed only at the adult stage (Ghaffary *et al.,* 2012). Overall phenotyping results showed the absence of any clear relationship between the presence of a particular D-chromosome and STB resistance. However, resistance in the "Langdon" genotype was found to be regulated by resistance genes located on the A- and B-genomes, which were expressed at different growing stages, such as genes on 3A and 7B that were expressed in seedlings and genes on 1B and 3B expressed at the adult stage. A gene(s) on chromosome 7A appeared to confer resistance at both stages. Resistance in the "Langdon" genotype seemed to be the result of an additive effect between all these genes. This again has been seen previously with many sources of resistance to STB disease in bread wheat carried by more than one *Stb* gene each conferring partial resistance. For example, four isolate specific *Stb* genes (*Stb6, Stb7, Stb12* and *Stb10*) were identified in the resistant bread wheat cultivar "KK4500" (Chartrain *et al.,* 2004, 2005a). Hence, in field trials, resistance to STB does generally appear as a quantitative trait, largely additive in nature. Through bi-parental mapping for STB resistance, quantitative resistance with additive effect was seen in both durum wheat (van Ginkel and Scharen, 1987, 1988; Berraies *et al.,* 2014) and bread wheat (Danon and Eyal, 1990; Jlibene *et al.,* 1994; Simon *et al.,* 1998).

High-throughput genotyping using the DArTseq™ system, that combined DArT markers with next-generation sequencing (NGS), of four pentaploid populations allowed the quantification of the genomic proportions of the A-, B-

and D-genome alleles inherited from the bread wheat. This technique has been successfully applied to high-throughput screening of genetically diverse plant materials (Ren *et al.,* 2015). Results suggested that the choice of the durum wheat parent plays a major effect in the retention of bread wheat alleles in the populations studied, with a higher retention rate of the A- and B-genome bread wheat alleles in the C3 population (32%) crossed to "Om Rabiaa 5" compared to the C4 population (17%) crossed to "Karim". Furthermore, a significate positive correlation was found between the mean percentage of the retained A- and the B-genome alleles and D-genome allele retention. Martin *et al.* (2011) also showed that lines derived from pentaploid crosses with higher levels of durum wheat A- and B-chromosome segments tended to retain fewer D-genome chromosomes. This finding suggests that the A- and B-genomes of hexaploid wheat have a higher capacity to retain unbalanced D-genome chromosomes than does the tetraploid wheat genome (Deng *et al.,* 2018).

Field based screening showed a very high level of resistance at the F_1 generation reflected by the complete absence of disease symptoms on all the screened plants whereas the backcross populations segregated for disease resistance. Resistant plants that had lost all D-genome were also identified in all the screened population. This does suggest that the resistance genes are mainly located on the A- and/or the B-genome of the bread wheat. In a similar study, the introgression of a chromosome segment from 4B from the bread wheat enhanced the resistance of the durum wheat to FCR (Ma *et al.,* 2012).

In addition, the identification of plant with different levels of resistance suggested that resistance in bread wheat could be controlled by more than one gene that act together conferring the complete immunity of bread wheat to this specific population of *Z. tritici*. This pathogen is known to be highly genetically diverse, induced by the several sexual reproduction cycles during the growing season of wheat (Kema *et al.,* 1996c, McDonald *et al.,* 1999), which can increase the risk of adaptation of the pathogen to overcome the resistance genes. The presence of this high genetic variability within the *Z. tritici* population again supports the hypothesis that the durable resistance of the bread wheat in Tunisia for over 40 years, is most probably quantitative.

A significant negative correlation between the D-genome allele retention and STB disease severity was obtained using a synthetic hexaploid parent

"Synthetic 6X" crossed to "Karim" suggesting the potential presence of an effective partial resistance gene in the *Ae. tauschii* D-genome donor of this synthetic. Zhu *et al.* (2016) demonstrated the potential use of *Ae. tauschii* for the improvement of FHB resistance in durum wheat. Chromosomes 1D and 5D from *Ae. tauschii* were found to carry genes for FHB resistance when they were individually or simultaneously added to "Langdon".

It is known that *Ae. tauschii* constitutes an important source of useful genes for wheat improvement with resistance genes to most of the damaging pests of wheat being identified, together with genes for physiological traits and abiotic stresses (see section 4.1.3. for examples).The exploitation of *Ae. tauschii* for durum wheat improvement has been very limited. Only a few targeted D-genome segments bearing genes of interest have been transferred from bread wheat into durum wheat, mainly due to the limited recombination undergone by *Ae. tauschii* in a cross with durum wheat in the presence of *Ph1* as compared to a cross with bread wheat that already carries the complete D-genome.

All 25 accessions of *Ae. tauschii* screened in this work were found to be completely immune to *Z. tritici* at both stages. The introgression of the D-genome of *Ae. tauschii* into durum wheat thus has considerable potential to enhance durum wheat resistance to STB disease (and possibly many other diseases). To generate a panel of durum wheats with different D-genome segments, the Langdon 5D (5B) disomic substitution line was used in a first cross to *Ae. tauschii* (selected for the absence of the *Ph1* gene on 5BL). Amphapaloid F_1 seeds were as haploid, but a low crossability level was found between the LND 5D (5B) substitution line and *Ae. tauschii*. This might have been caused by the presence of dominant alleles on chromosomes 7A and 4B and responsible for inhibiting the crossability with *Ae. tauschii* (Zhang *et al.*, 2008). Several translocations between the D-genome and the A- or B-genomes plus a number of RobT were found in the different progeny with plants mainly carrying a total of 28 or 29 chromosomes. The univalent state of the 5B chromosome and the D-chromosomes promoted the production of four different B-D RobT, involving either arm of chromosome 5B with one of the 3DL, 5DS or 5DL chromosome arms, generating the 5BS-5DL, 3DL-5BL, 5DL-5BL and, 5DS-5BL translocations. This centromeric breakage of univalent chromosomes has previously been used to generate and to manipulate translocations of alien

chromosome arms into wheat (Lukaszewski, 1993, 1997). Once homozygous, these translocation lines can be phenotyped for many traits, including STB disease resistance. The use of the LND 5D (5B) substitution line thus promises to be an effective strategy to generate durum wheat/D-genome introgression lines. Although time consuming and technically demanding, the possible benefits of this strategy could be far reaching for durum breeding.

To date, strategies for the introgression of the D-genome into durum wheat have mainly relied on the use of the Langdon D-genome disomic substitution lines (Joppa and Williams, 1988). Hence, the transfer of D-genome translocations found to be present in hexaploid wheat/alien introgression lines could prove be an alternative, and possibly for effective, strategy for D-genome introgression into durum wheat. Genomic translocations were traced from one generation to another, together with new translocations which appeared to be occurring only in the lines still containing an alien chromosome. This could be explained either by the presence of four genomes together (A-, B-, D- and T-genome) in one cell, with an unequal chromosome number that can promote an abnormal meiotic behaviour leading to homoeologous paring and therefore new genomic translocations. Another hypothesis to be considered is the potential presence of a *Ph1* suppressor gene on the retained T-segment present. In fact, *Am. muticum* species is known to harbour a gene(s) that promotes pairing between homoeologous chromosomes and suppresses the effect of *Ph1* in hybrids with allopolyploid wheat (Dvořák, 1972; Dover and Riley, 1972).

During this study, most intergenomic exchanges observed involved the A- and D-genomes. Because of their preferential association at metaphase I in interspecific hybrid combinations, it is generally assumed that these wheat genomes are very closely related (Fernández-Calvín and Orellana, 1994; Maestra and Naranjo, 1998). Out of the A/D translocations, the "A-d" type was found to be the most frequently formed and retained. This result is consistent with the study of Sanchez-Moran *et al.* (2001) who demonstrated the frequent occurrence of such translocations from among all the A- and D-genome translations found in progeny generated using the *ph1b* genotype. Additionally, it is well established in cereals that chiasmata tend to be formed most frequently in the distal chromosome regions (Lukaszewski, 1995), which concurs quite well with the predominant distal position of exchange points reported here. In

the present study, only the "A-d" translocation type was successfully introgressed into the tetraploid background of both durum wheat varieties, suggesting that the smaller the D-translocation, the higher the chances of it being retained and introgressed into durum.

Results also suggested the higher tolerance of "Karim" to the presence of genomic translocations compared to "Om Rabiaa 5". This higher tolerance of "Karim" genotype for the presence of genomic rearrangements/introgressions was seen in all generations. The choice of the durum wheat genotype therefore appears to play a major role for successful alien genome introgression in durum wheat. This must be taken into consideration in any future programme, e.g. it would wise to initially use several durum genotypes to try and ensure the use of at least one with a higher tolerance to alien introgressions.

Where chromosome segments are introgressed into either hexaploid or durum wheat, it is essential that the segments have no detrimental effects on plant growth, etc. Nine of the BC_1-F_3 lines were tetraploid and carried simultaneous homozygous introgressions of both the D- and the T-genomes. These genotypes had vernalisation requirements very similar to the durum parent (three to four weeks). In glasshouse conditions, the plants showed a normal growth cycle as for a spring durum wheat, had a durum head morphology and were completely fertile, suggesting that no negative effect on plant vernalisation, vigour, growth cycle or fertility was associated with the D- and T-genome segments introgressed.

Wheat wild relatives are considered to harbour a vast genetic variability that could be used for wheat improvement by the introgression of important agronomical traits (Friebe *et al.*, 1996; Jauhar and Chibbar, 1999; Qi *et al.*, 2007; Schneider *et al.,* 2008). Thus the lines developed in this work, which contained *Am. muticum* segments, have the potential to contain important new genetic variation. For example, *Am. muticum* is known to be a useful source of resistance to fungal diseases of wheat (Panayotov *et al.,* 1997; Eser, 1998). Phenotyping of the amphidiploid Aurotica (AABBTT) (Zhirov, 1989), showed it to be resistant to rust diseases, powdery mildew, fusariosis, and septoriosis and also environmental stresses such as frost resistance (Iefimenko *et al.*, 2015). Hence, tetraploid *Am. muticum* introgression lines have the potential to enhance

the level of disease resistance in durum wheat and also traits related to yield potential.

Mc-FISH analysis, showed that one of the D-genome segments introgressed into the tetraploid background of "Om Rabiaa 5" could potentially be the telomeric region of the 3DS chromosome arm translocated with the short arm of chromosome 5A. However, the small D-genome segment introgressed into the tetraploid genomic background of "Karim" was too small in size and couldn't be characterised. Cytological methods such as mc-GISH and mc-FISH can enable the identification of deletions, translocations, introgressed chromatin fragments, and translocation breakpoints (Le *et al.,* 1989; Schwarzacher *et al.,* 1989; Friebe *et al.,* 1993). As with the small segment above, cytogenetic-based approaches for introgression identification/characterisation can have a limited resolution and therefore the use of molecular markers should be considered as well. The information generated through molecular markers alone is insufficient to validate introgression lines that show chromosome deletions, additions, or translocations. Thus, it is essential to apply cytological techniques, such as mc-GISH or mc-FISH, which provide a more systematic approach for analysing complex chromosomal complements (Eberhard *et al.,* 2010) and combine it with characterisation using molecular markers once the introgressions are homozygous in a stable genomic background.

Known that wheat sub-genomic translocation occur often during the process of developing wheat/alien introgression lines, these genomic translocation and particularly the ones that involve the D-genome can be of use for the introgression of the D-genome into durum wheat. In the present study (chapter 5) we demonstrated that crossing the hexaploid wheat/*Am. muticum* introgression harbouring translocation involving the D-genome to durum wheat is a novel crossing strategy that allows making use of these D-segments in durum wheat breeding.

7 Conclusions

The genetic variability derived from bread and durum wheat and transferred into pentaploid hybrids has the potential to improve disease resistance, abiotic tolerance, and grain quality, and to enhance agronomic characters (Padmanaban *et al.*, 2017a). Little emphasis has been placed on developing efficient methods to incorporate these pentaploid hybrids into commercial breeding practices. This is mainly due to the limiting factors that affect the production of a viable F_1 hybrid (reviewed in Padmanaban *et al.*, 2017a). For instance, the low F_1 seed obtained (Sharma and Gill, 1983), poor germination rate (Kihara, 1982; Sharma and Gill, 1983), seedling abnormality (Tsunewaki, 2004), pollen viability (Bhagyalakshmi *et al.*, 2008) and progressive hybrid necrosis (Chu *et al.*, 2006) of the F_1 progeny.

In the present thesis we showed that variable F_1 pentaploid hybrid seeds were generated using different combinations of pentaploid crosses, involving either common wheat, Synthetic hexaploid wheat, LND-DS lines or hexaploid alien introgression lines carrying genomic translocations for durum wheat improvement. Pentaploid crosses, between resistant hexaploid genotypes carrying *Stb* genes with susceptible durum wheat genotypes, combine the steps of *Stb* gene transfer, the introgression of genetic variability from the bread wheat into durum wheat while developing mapping populations that potentially enable the identification of markers closely linked to resistant genes that can be used for durum wheat breeding to STB disease. The *Stb* genes *Stb6*, *Stb7*, *Stb12* and *Stb17* were found to be ineffective in a durum wheat background. However,

resistant tetraploid plants were identified, suggesting the potential presence of resistance genes in the bread wheat that can be used for durum wheat improvement.

Viable F_1 pentaploid progeny were generated using different LND-DS lines. Cytogenetical analysis showed that pentaploid crosses involving LND-DS lines promote the retention of D-chromosomes in the advanced backcrossing generations from the F_1 pentaploid to durum wheat. Furthermore, viable F_1 pentaploid progeny were generated from hexaploid wheat/*Am. muticum* introgression lines carrying genomic translocation. Further crossing and backcrossing of the F_1 pentaploid, showed the potential to establish tetraploid populations and the introgression of either the genomic segment or the trait of interest. Hence, our results suggest that a pentaploid crossing strategy can be successfully applied in durum wheat breeding as a means of durum wheat improvement, using not only bread wheat/durum wheat crosses but also in crosses involving LND-DS lines or hexaploid alien introgression lines carrying genomic rearrangements.

Introgression approaches using either the bread wheat D-genome or the direct use of the D-genome donor *Ae. tauschii* have the potential to enhance durum wheat genetic variability and enable the study of the effect of smaller D-genome segment introgressions (that ideally cover the whole of the D-genome) into durum wheat, compared to the only other available stocks, i.e. the Langdon disomic D-genome substitution lines developed by Joppa and Williams (1988).

Furthermore, the introgression of D-genome translocations present in the genetic background of hexaploid/*Am. muticum* introgression lines through crossing to durum wheat was shown to be an efficient strategy to make use of these D-genome segments. To the best of our knowledge, no previous work has taken place to purposely introgress D-genomic rearrangements and/or *Am. muticum* segments, present in hexaploid wheat/alien introgression lines, into durum using pentaploid crosses. Further backcrossing to the tetraploid parent has the potential to eliminate any univalent D-chromosomes and to isolate the alien segment into the tetraploid background in a homozygous state. The presence of *Am. muticum* segment in the introgression lines promoted the occurrence of new genomic translocations in the backcross generations. In the small study conducted here, several small D-genome segments were

introgressed into a durum background. D-genome rearrangements with both the A- and B-genomes have been observed in the background of many of the introgression lines produced at the Nottingham/BBSRC Wheat Research Centre. These introgression lines include those produced with several alien species including *Am. muticum*, e.g. *Ae. speltoides*, *Th. bessarabicum*, *Th. elongatum*, etc. The transfer of these D-genome segments into durum has the potential to revolutionise durum breeding. It might even be possible to use an introgression system to generate small inter-genomic D-genome rearrangements from a hexaploid wheat carrying genes of interest on the D-genome and then to transfer the small D-genome segments into durum wheat.

Overall results showed that in all the pentaploid cross combinations used, the parental choice has played a major role. For instance, a higher genetic variability inherited from the bread wheat parent, was captured using the durum wheat genotype "Om Rabiaa 5", whereas the durum wheat genotype "Karim" appeared to be very receptive/tolerant to the presence of genomic translocations when used for the introgression of alien genome segments. In crosses involving hexaploid wheat with LND-DS lines, it was the choice of the hexaploid parent which appeared to have more effect on the number of F_1 seed obtained.

8 Perspectives and future work

- Field based phenotyping of four pentaploid populations to STB disease showed a segregation for disease resistance (Chapter 3). Taking into consideration the presence of genetic recombination between the A- and B-genomes of the bread wheat and the durum wheat and its absence in the D-genome, the genotyping data of these populations will be used to contract genetic maps of the A- and B-genomes (e.g. Zhang *et al.*, 2012). Therefore, in combination with the phenotyping data collected, mapping studies for resistance QTL to STB disease in these populations will be undertaken. The identification of these QTL will then be used in durum wheat breeding to give an idea of the genomic region in wheat responsible for *Z. tritici* specificity in either the durum or bread wheat cultivars in Tunisia.
- Effective transfer of *Stb* genes located on the D-genome of bread wheat into susceptible durum wheat can be achieved using three-way crosses involving the use of LND-DS lines as a bridge between the bread and the durum wheat parents. Screening for the retention of these genes using cytogenetic approaches to visualise the presence of the D-genome, combined with closely linked molecular markers should be considered particularly in earlier generations of a backcrossing programme.
- The durum wheat Langdon genotype showed a good level of resistance to STB disease at the seedling as well as the adult stage. The crossing of Langdon to a susceptible durum wheat could help mapping the resistance in this genotype and make it of use for durum wheat improvement. However, we should take in consideration the presence of susceptibility genes to the durum wheat specific isolates of the pathogen, as was seen in the phenotypic data generated in the present thesis.
- The introgressed *Ae. tauschii* D-genome segments in the progeny of LND 5D (5B)/*Ae. tauschii*//Om Rabiaa 5 crosses were mainly the result of Robertsonian translocations brought about by the centromeric breakage-fusion of univalent chromosomes in double monosomic plants (Chapter 4). D-genome translocations occurred mainly with the 5B chromosome as a result of its univalent state in all of the BC_1-F_1 lines and 30% of the BC_1-F_2 lines. Based on this, besides the 4D (4B), 5D (5A) and 3D (3B) substitution

lines that are maintained with a telosome or monosome of the substituted A- or B-genome chromosomes, the use of other LND-DS lines could also be considered for *Ae. tauschii* introgression into durum wheat.

- *Ae. tauschii* accessions showed a complete immunity to STB disease caused by *Z. tritici* durum wheat specific isolates. Once the introgressed D-genome segment is homozygous, the introgression lines carrying different D-genome segment can be phenotyped for STB disease.
- It was noticed that in the presence of *Am. muticum* T-genome segment in the WMI lines crossed to durum wheat, new translocations occurred in the progeny produced (Chapter 5). Taking into consideration that *Am. muticum* harbours a *Ph1* suppressor gene, the potential presence of this gene in the introgressed segment should be studied further. Other studies support the idea that the presence of four genomes in one cell in an unbalanced chromosome number can also promote the occurrence of novel genomic rearrangement. Verification of this hypothesis could be achieved by following the same crossing strategy but using introgression lines carrying chromosome segment of other alien species, such as *Th. bessarabicum* (2n=2x=14, JJ or E^bE^b).
- Genotypic characterisation of the homozygous D-genome segment and/or T-genome from the WMI lines using molecular marker should be carried out. These lines can be used to phenotype a wide range of traits such as disease resistance.
- Another crossing strategy for the introgression of the bread wheat D-genome into durum wheat in small segments, that should be considered, would be crossing a *ph1b* hexaploid wheat such as Paragon or Chinese Spring *Ph1* mutants to *ph1c* tetraploid wheat such as Creso or Cappelli *Ph1* mutants. Translocations involving the D-genome can then be expected in the gametes of the double mutant *ph1b/ph1c* F_1 pentaploid. Top crossing of the F_1 to normal durum wheat followed by a few backcrosses would further induce elimination of univalent D-chromosome. D-introgression can be traced using mc-GISH through the generations until homozygous in tetraploid background.

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Appendix

Appendix 1: 5% Sodium hypochlorite (ClNaO)

Sodium hypochlorite (ClNaO)……..5 ml

Water……...95 ml

Tween 20…….…..1-2 drops

Appendix 2: Extraction buffer (for 100 ml)
0.1M Tris-HCl (pH 7.5)……...10 ml

0.05M EDTA (pH 8.0)…….…10 ml

10% SDS……...12.5 ml

Water…….67.5 ml

Appendix 3: 6M ammonium acetate (for 50 ml)
6M $NH_4C_2H_3O_2$……...…...23.124g
Water…….50 ml
Store solution at 4°C

Appendix 4: Cell digestion enzyme (for 100ml)

Enzyme solution made on ice by mixing the enzyme in 1X Citric buffer**

Pectolyase Y-23 (1% w/w)…….0.1 g

Cellulase Onozuka R-10 (2% w/w)……0.2 g

1X Citric Buffer (pH 5.5)**....……...9.7 g

The enzyme solution was divided into 20 µl aliquots and stored at -20°C.

****For 50ml of 5x Citric Buffer**

Water……50 ml

Sodium Citrate (50mM)…….…0.735 g

0.5M EDTA (50mM)…….5 ml

Adjust pH to 5.5 using Citric acid monohydrate powder, add, filter solution and stored at room temperature

****For 100ml of 1x Citric Buffer**

5x Citric Buffer……....20 ml

Water……80 ml

Add the solution with water up to 50 ml; dissolve and mix well. Sterilize the solution by using syringe with filter (0.2um) and store at room temperature or in the fridge.

Appendix 5: Mc-GISH probe reaction solution

DNA (>200ng/µl)……..……...2 µl

10x Nick translation buffer…….……2 µl

Non-labelled dNTPs……..….....2 µl

Labelled dNTP (1mM)……...…...0.5 µl

DNA polymerase I (10U/µl)…….…..…….4 µl

DNase**...……..…...0.5 µl

*DNase (100 mU/µl)…….…….....5 µl

*50% glycerol…….....95 µl

Water…….....up to 20 µl

Appendix 6: Nick translation reaction of mc-FISH

Plasmid DNA (pSc119.2 or pAs.1).....2 µl

10x Nick translation buffer ……...…..2 µl

Non-labelled dNTPs…….....2 µl

Labelled dNTP (1mM)…….....….0.5 µl

DNA polymerase I (10U/µl).....4 µl

DNase* …….…….....0.5 µl

*DNase (100 ...U/µl)….....5 µl

*50% glycerol…….....95 µl

Water up to…….....20 µl

Appendix 7: SS DNA (140 µg/ml) for 50 ml

50X TAE (pH 6.3)…….....5 ml

SS DNA (10mg/ml)…….….0.7 ml

Water …….....44.3 ml

Store at 4°C

Appendix 8: 3M Sodium Acetate (NaOAc, pH 5.2) for 100 ml

Sodium acetate (m.w.-136.08)……..40.8 g

Water…….…….....100 ml

Adjust pH to 5.2 and sterilise the solution by 0.22 µm membrane filter

Appendix 9: **2xSSC+1×TE solution** for 20 ml

20×SSC*…….....2 ml

10×TE**…….....2 ml

Water……...…….16 ml

***2× SSC** (for 100ml)

20x SSC……10 ml

Water……...….90 ml

****10 × T.E.**

100mM Tris

10mM EDTA

pH7.5

****1 × T.E.**

10xT.E …….....10 ml

Water…….…90 ml

Appendix 10: Single colour-GISH probe mix (10 µl per slide)

Am. muticum (green)……...…....1 µl

2xSSC in 1x TE…….…….....9 µl

Appendix 11: Multicolour-GISH probe mix (10 µl per slide)

Ae. uratu DNA probe (green)……..1.5 µl

Ae. tauschii DNA probe (red)…….…….3 µl

Blocking *Ae. speltoidies* DNA……....4 µl

2xSSC in 1x TE…….....1.5 µl

Appendix 12: Multicolour-FISH probe mix (10 µl per slide)

pSc119.2 DNA probe (green)…….0.1.5 µl

pAs.1 DNA probe (red)…….……...3 µl

2xSSC in 1x TE……..…1.5 µl

Appendix 13: 1× TAE buffer (pH 8.6)

Tris base……..….40 mM

Acetate…….…….20 mM

EDTA……...…..1 mM

Appendix 14: *Stb* genes SSR markers

***Stb* genes**	**Marker name**	**Chromosome**	**Annealing temperature**	**Germplasm source**
Stb5	*gwm44*	7DS	58^{o}C	Synthetic 6X
Stb6	*gwm369*	3AS	54^{o}C	Synthetic 6X, Kavkaz-K4500
Stb7	*wmc313*	4AL	53^{o}C	Kavkaz-K4500
Stb10	*wms848*	1Dc	52^{o}C	Kavkaz-K4500
Stb12	*wmc219*	4AL	55^{o}C	Kavkaz-K4500
Stb16q	*wmc494*	3DL	51^{o}C	SH M3
Stb17	*hbg247*	5AL	60^{o}C	SH M3

Appendix 15: D-genome specific SSR markers

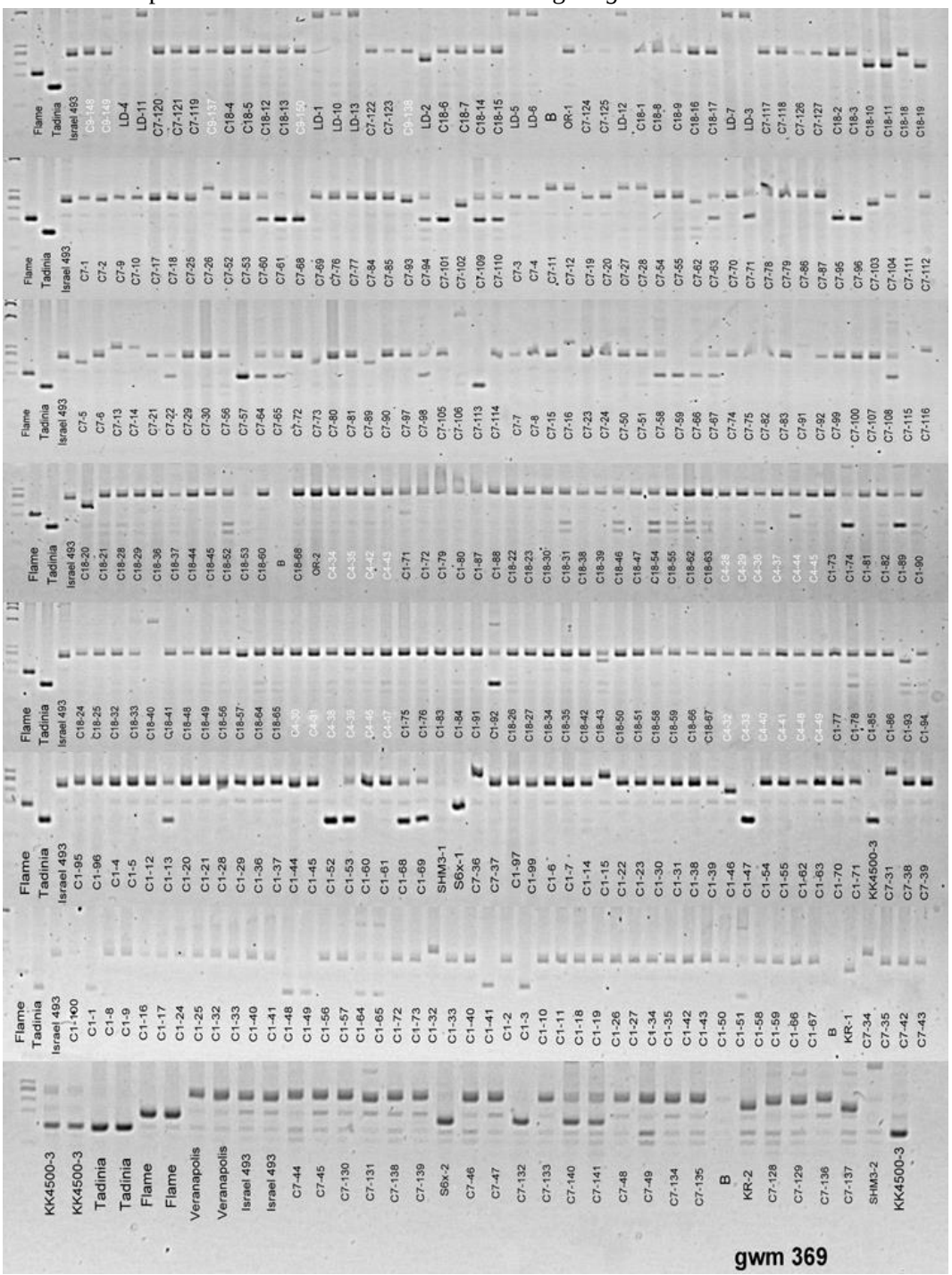
Marker name	Locus	Annealing temperature	Sequence*
wmc432	1DL	51^{o}C	**F:** ATGACACCAGATCAGCAC **R:** AATATTGGCATGATTACACA
cfd82	1Dc	60^{o}C	**F:** GCTGATGCTGCTGTAAGTGC **R:** TGAAGAATACAATGGCAGCAA
cfd32	1DL	60^{o}C	**F:** CAACACAACCACAATTTCCG **R:** CTCAGGGAGGTCATGCAGAG
wmc603	2DS	61^{o}C	**F:** ACAAACGGTGACAATGCAAGGA **R:** CGCCTCTCTCGTAAGCCTCAAC
wmc18	2Dc	61^{o}C	**F:** TGGGGCTTGGATCACGTCATT **R:** AGCCATGGACATGGTGTCCTTC
cfd233	2DL	60^{o}C	**F:** GAATTTTTGGTGGCCTGTGT **R:** ATCACTGCACCGACTTTTGG
cfd141	3DS	60^{o}C	**F:** CGTAAAGATCCGAGAGGGTG **R:** TCCGAGGTGCTACCTACCAG
barc42	3Dc	52^{o}C	**F:** GCGACTCCTACTGTTGATAGTTC **R:** GCGTTCTTTTATTACTCATTTTGCAT
barc270	3DL	52^{o}C	**F:** GCGCATTGTGACAGGTGAAC **R:**GGAGGGAGTACTTGGTTATTAGGGT
wmc457	4DS	61^{o}C	**F:** CTTCCATGAATCAAAGCAGCAC **R:** CATCCATGGCAGAAACAATAGC
barc334	4Dc	57^{o}C	**F:** ATCCGCGTGTCAAACTTCTTCC **R:** GGGCTGGCTGGGCTAAATG
cfd84	4DL	60^{o}C	**F:** GTTGCCTCGGTGTCGTTTAT **R:** TCCTCGAGGTCCAAAACATC
cfd18	5DS	60^{o}C	**F:** CATCCAACAGCACCAAGAGA **R:** GCTACTACTATTTCATTGCGACCA
cfd78	5Dc	60^{o}C	**F:** ATGAAATCCTTGCCCTCAGA **R:** TGAGATCATCGCCAATCAGA
barc110	5DL	50^{o}C	**F:**CCCGAACAATGGCTTTGGTGTCGTAAT **R:** CATGGTGACGGCAAGTGTGAGGT
cfd49	6DS	60^{o}C	**F:** TGAGTTCTTCTGGTGAGGCA **R:** GAATCGGTTCACAAGGGAAA
cfd287	6Dc	60^{o}C	**F:** TCAAGAAGATGCGTTCATGC **R:** GGGAGCTTTCCCTAGTGCTT
cfd95	6DL	60^{o}C	**F:** AATCCTGACTTTAAAGCCTTTCC **R:** CATCTGTATGATATTTTGGAGGTCA
cfd46	7DS	60^{o}C	**F:** TGGTGGTATAGTCGTTGGAGC **R:** CCACACACACACACCATCAA
cfd14	7Dc	60^{o}C	**F:** CCACCGGCCAGAGTAGTATT **R:** TCCTGGTCTAACAACGAGAAGA
barc235	7DL	52^{o}C	**F:** GCGCTCACCCTCCTACACTTCCTA

	R: GCGCAAGTCTGTCAAAGCCTAA

*F: Forward, R: Reverse.

Supplementary materials

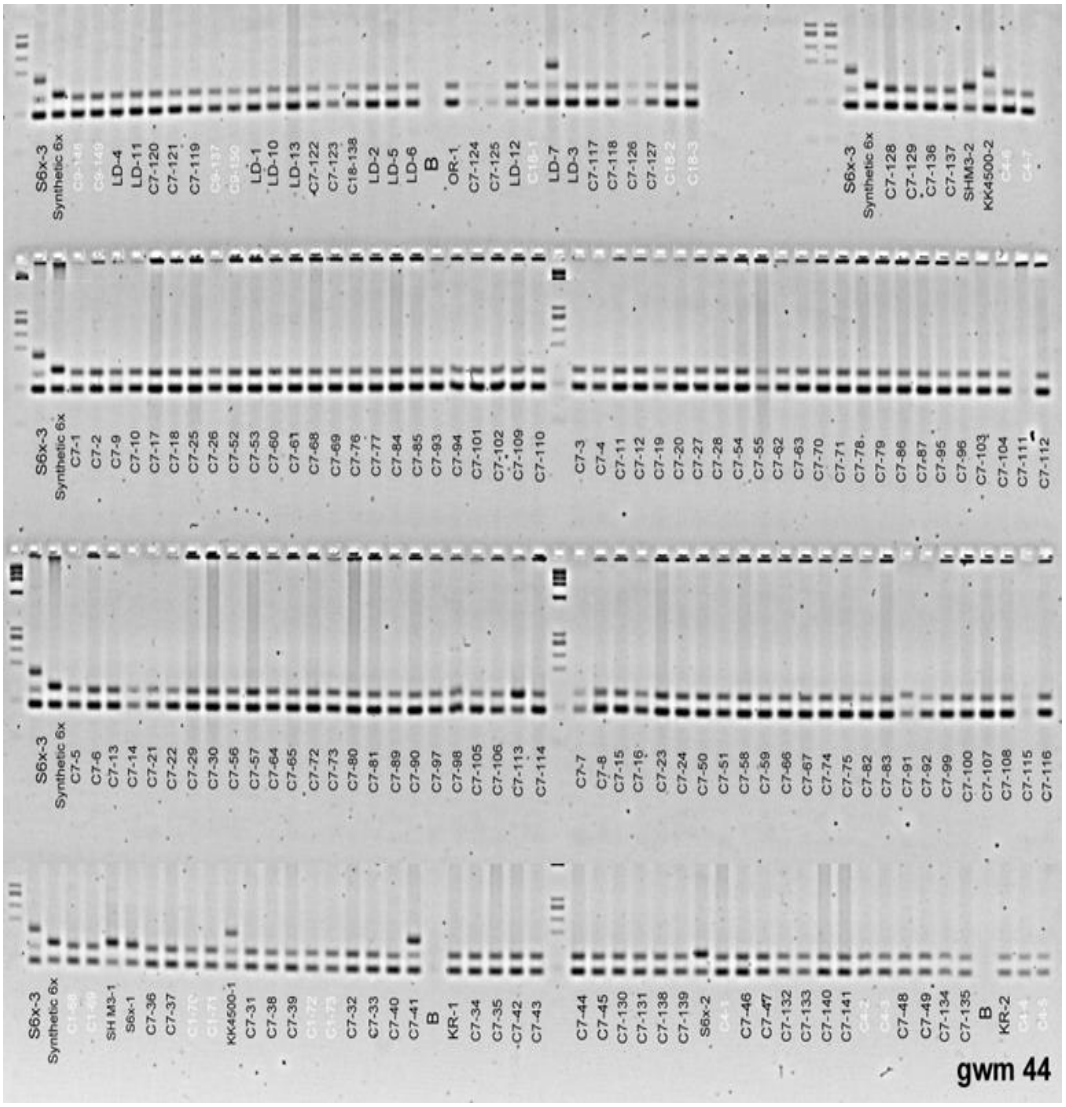
Supplementary material 1. Agarose gel screening of the C1, C7 and C18 populations for the presence of the *Stb6* using *gwm369* SSR marker.



*Negative control: Flame, Israel493 and Karim (KR)

* Positive control: Tadinia and KK4500

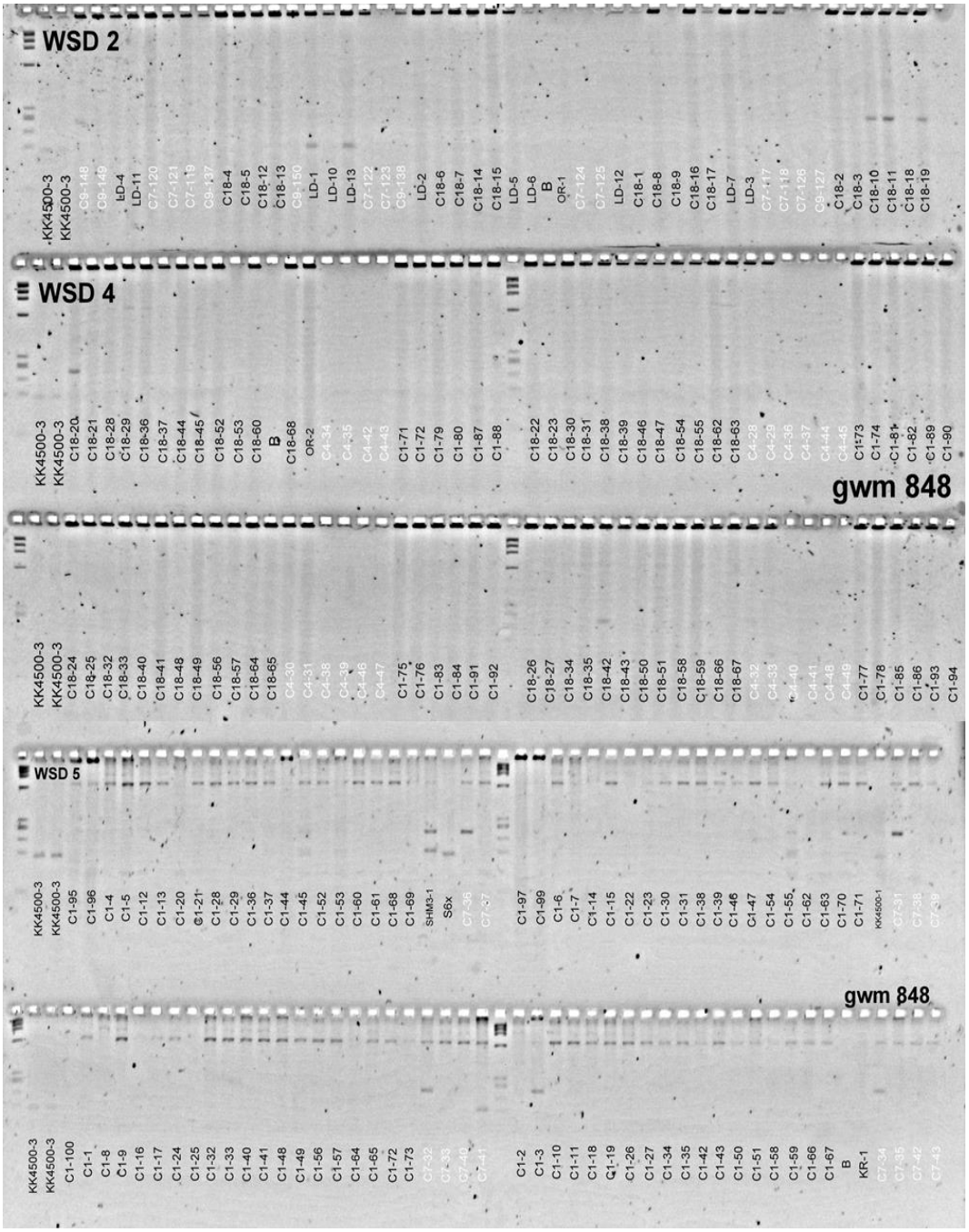
Supplementary material 3. Agarose gel screening of the C7populations for the presence of the *Stb5* using *gwm44* SSR marker.



*Negative control: SH M3 and Karim (KR)

* Positive control: S6X-3

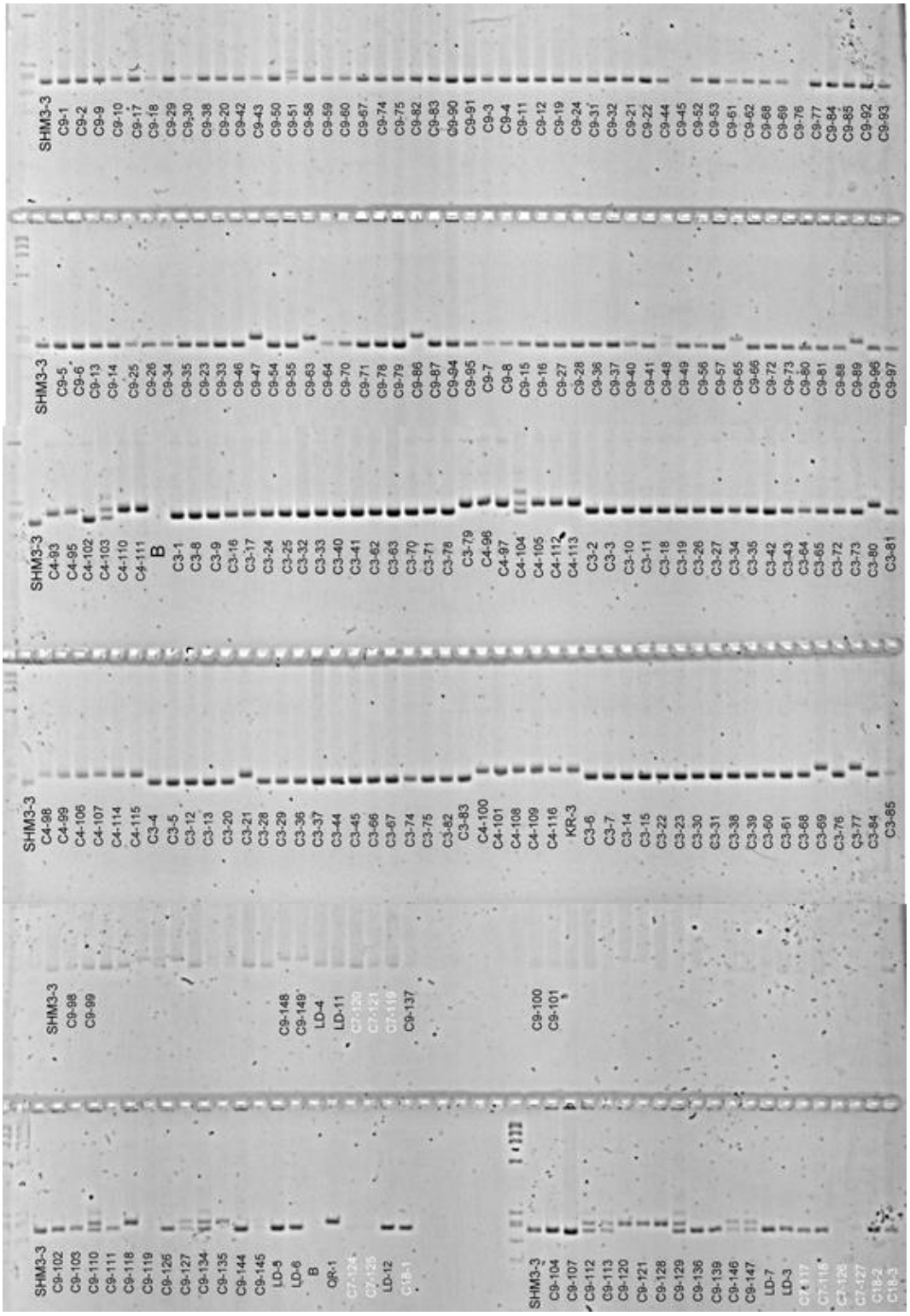
Supplementary material 3. Agarose gel screening of the C1 and C18 populations for the presence of the *Stb10* using *gwm848* SSR marker.



*Negative control: Karim (KR)

* Positive control: KK4500

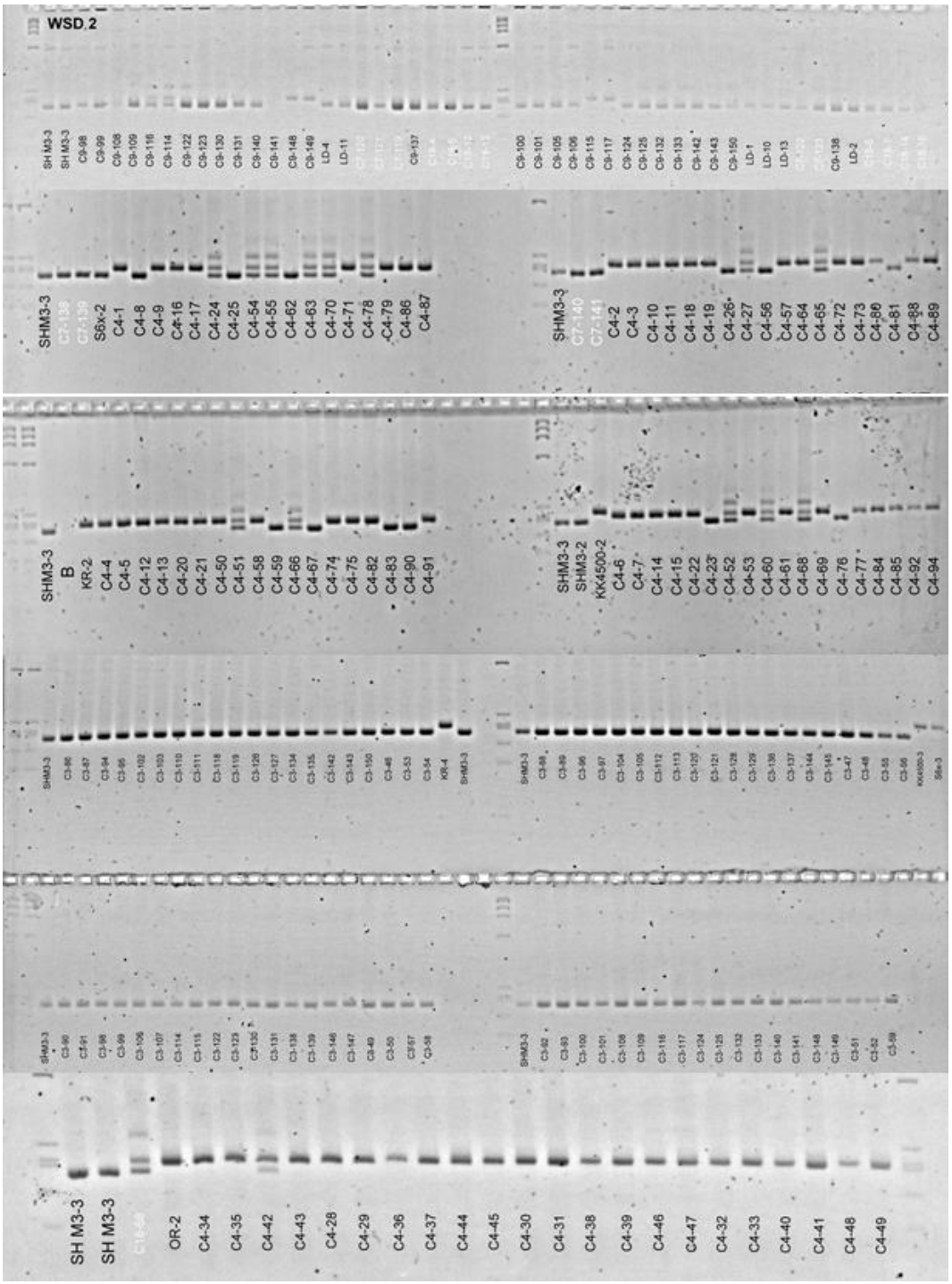
Supplementary material 4. Agarose gel screening of the C1 and C18 populations for the presence of the *Stb17* using *hbg247* SSR marker.



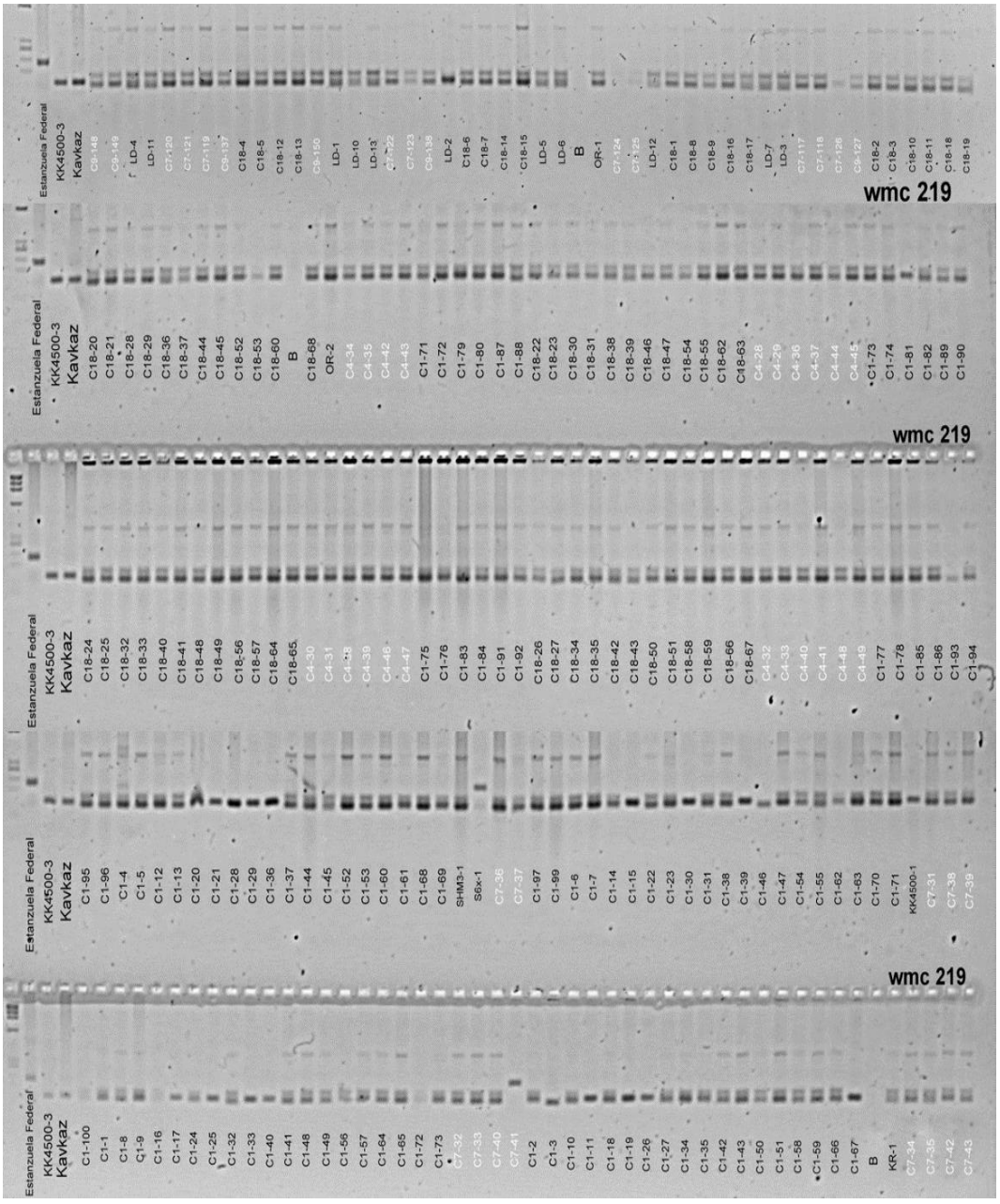
* Negative control: Om Rabiaa 5 (OR), Karim (KR)

* Positive control: SH M3

Supplementary material 4. (continue).



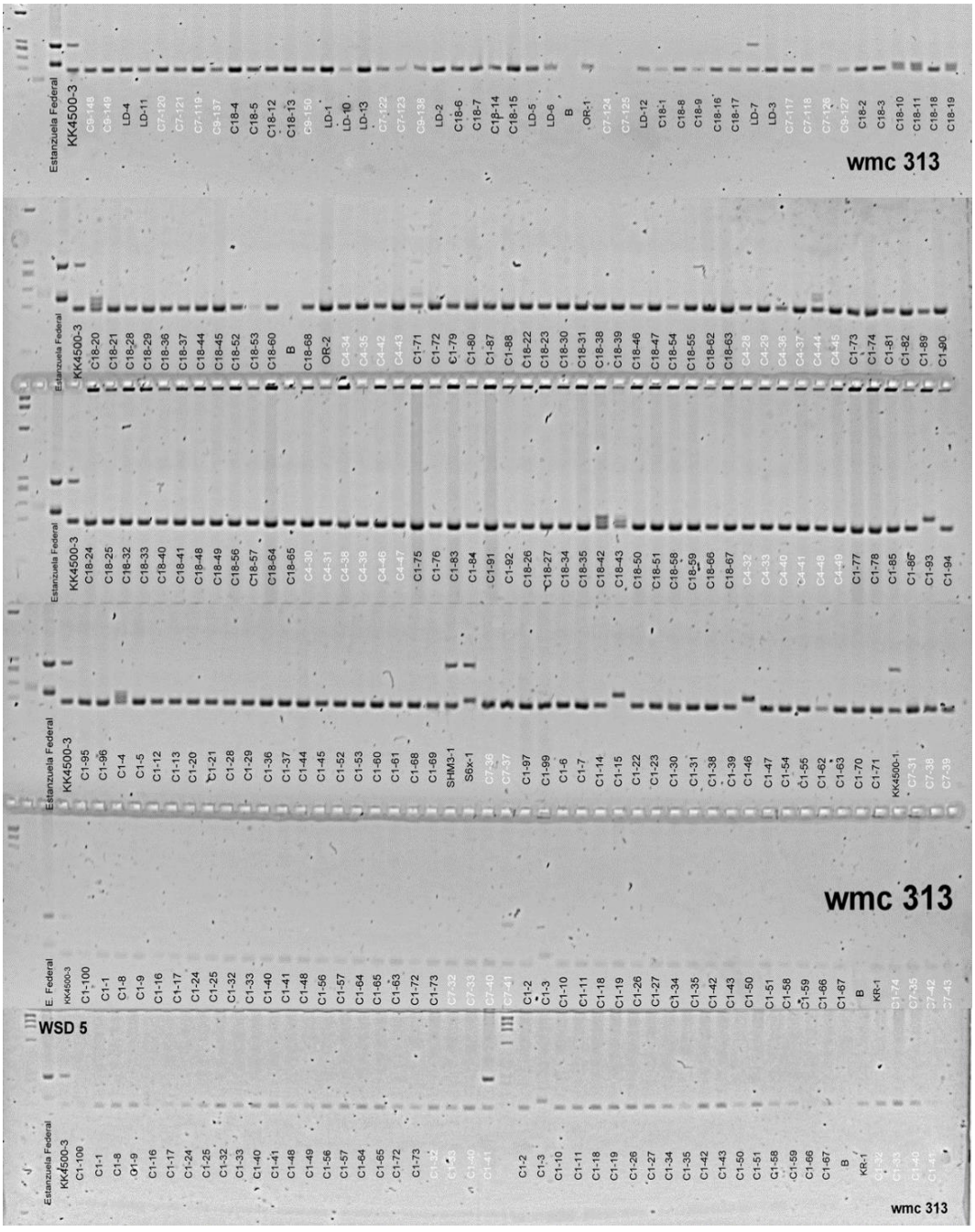
Supplementary material 5. Agarose gel screening of the C1 and C18 populations for the presence of the *Stb12* using *wmc219* SSR marker.



* Negative control: Estanzuela Federal

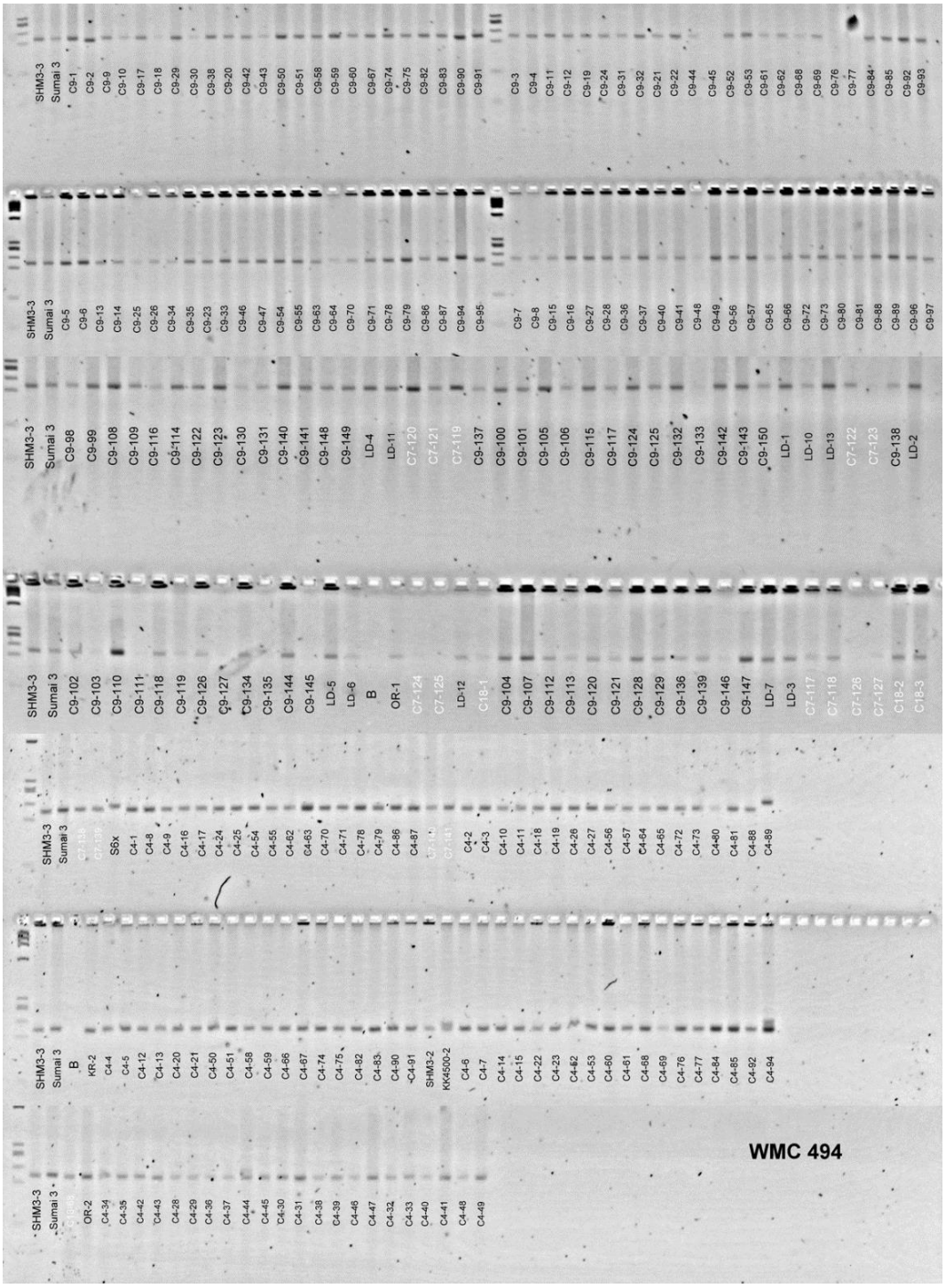
* Positive control: KK4500

Supplementary material 6. Agarose gel screening of the C1 and C18 populations for the presence of the *Stb7* using *wmc313* SSR marker.



* Negative control: Estanzuela Federal
* Positive control: KK4500

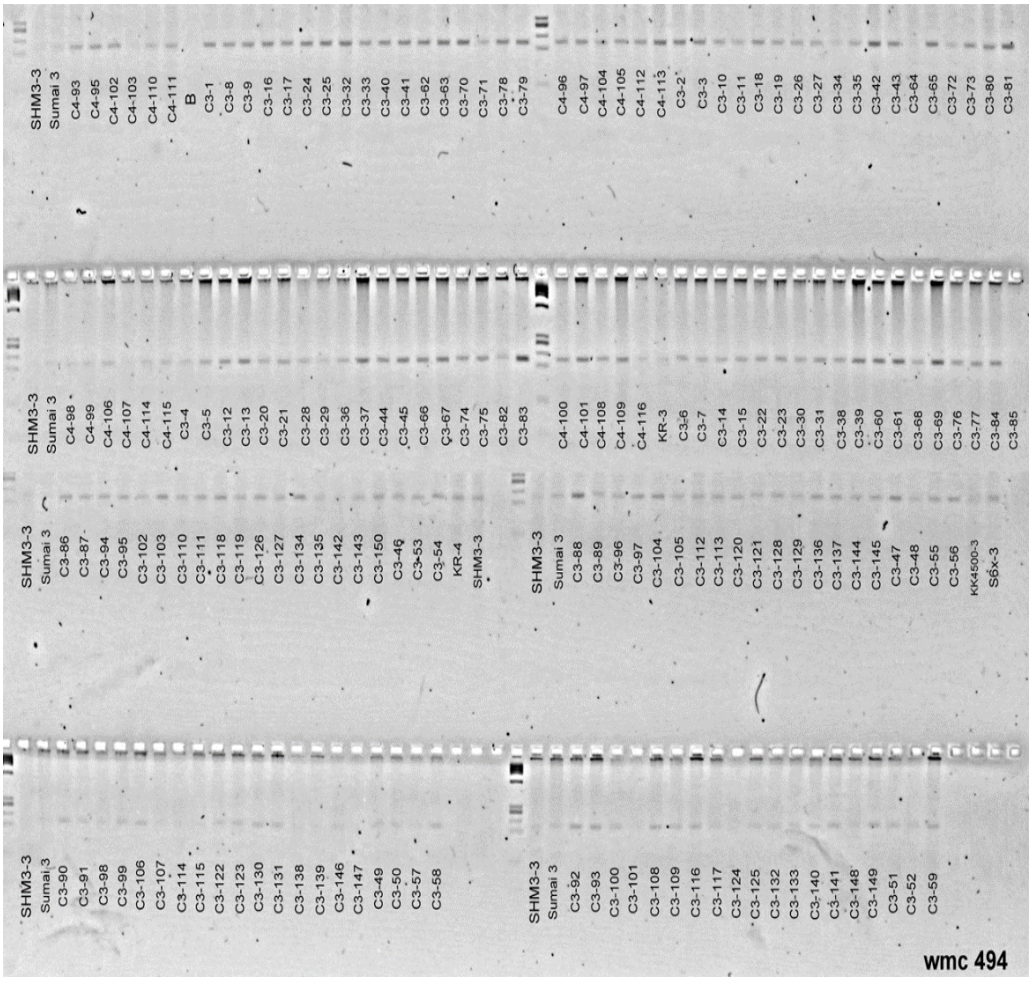
Supplementary material 7. Agarose gel screening of the C4 and C9 populations for the presence of the *Stb16* using *wmc494* SSR marker.



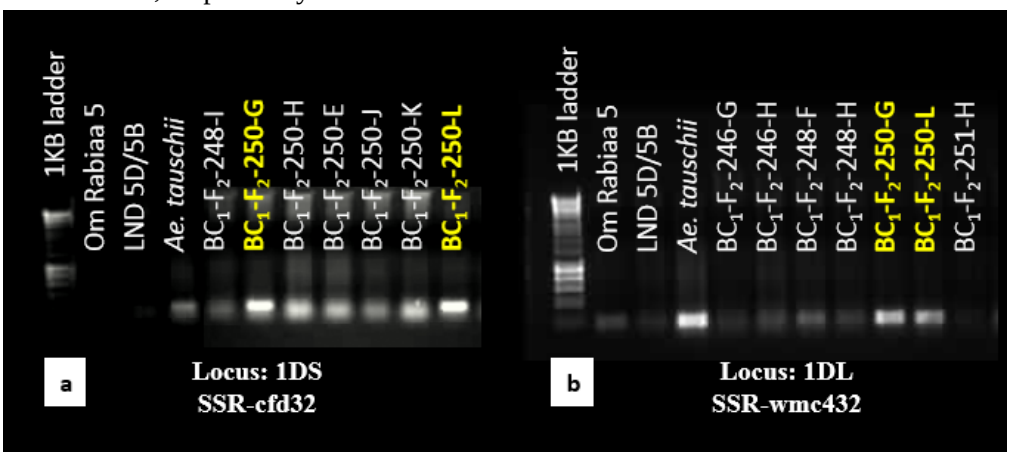
* Negative control: Sumai-3

* Positive control: SH M3

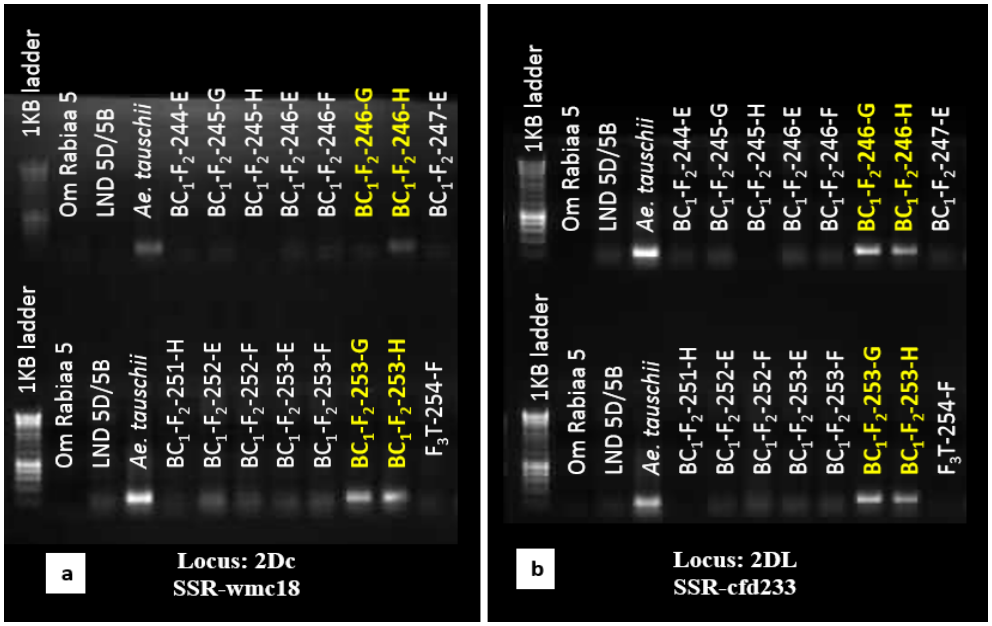
Supplementary material 7. Continue.



Supplementary material 8. Agarose-gel shows the presence of the 1DS (a) and 1DL (b) locus in the BC_1-F_2-250-G and -L sister lines (yellow) using the *cfd32* and *wmc432* SSR markers, respectively.

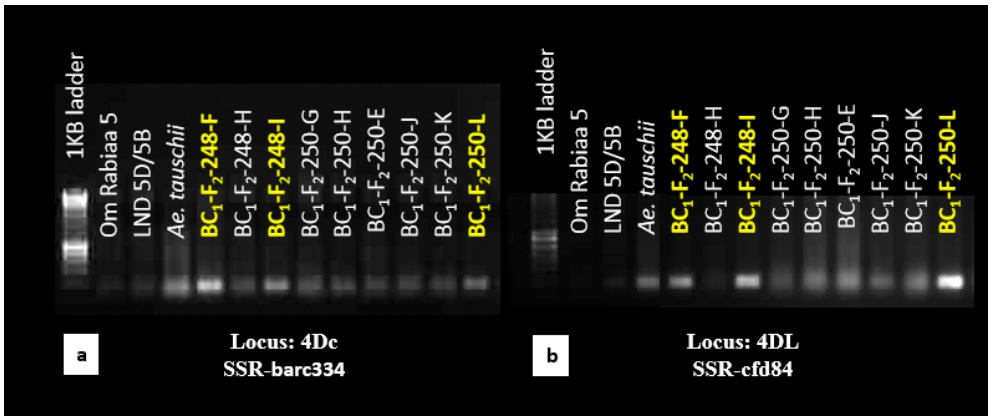


Supplementary material 9. Agarose-gel using the *cwmc18* (a) and *cfd233* (b) SSR markers showing the presence/absence of the 2Dc (a) and 2DL (b) locus, respectively, in the four BC_1-F_2-246-G and -H and the BC_1-F_2-253-G and -H lines (yellow).

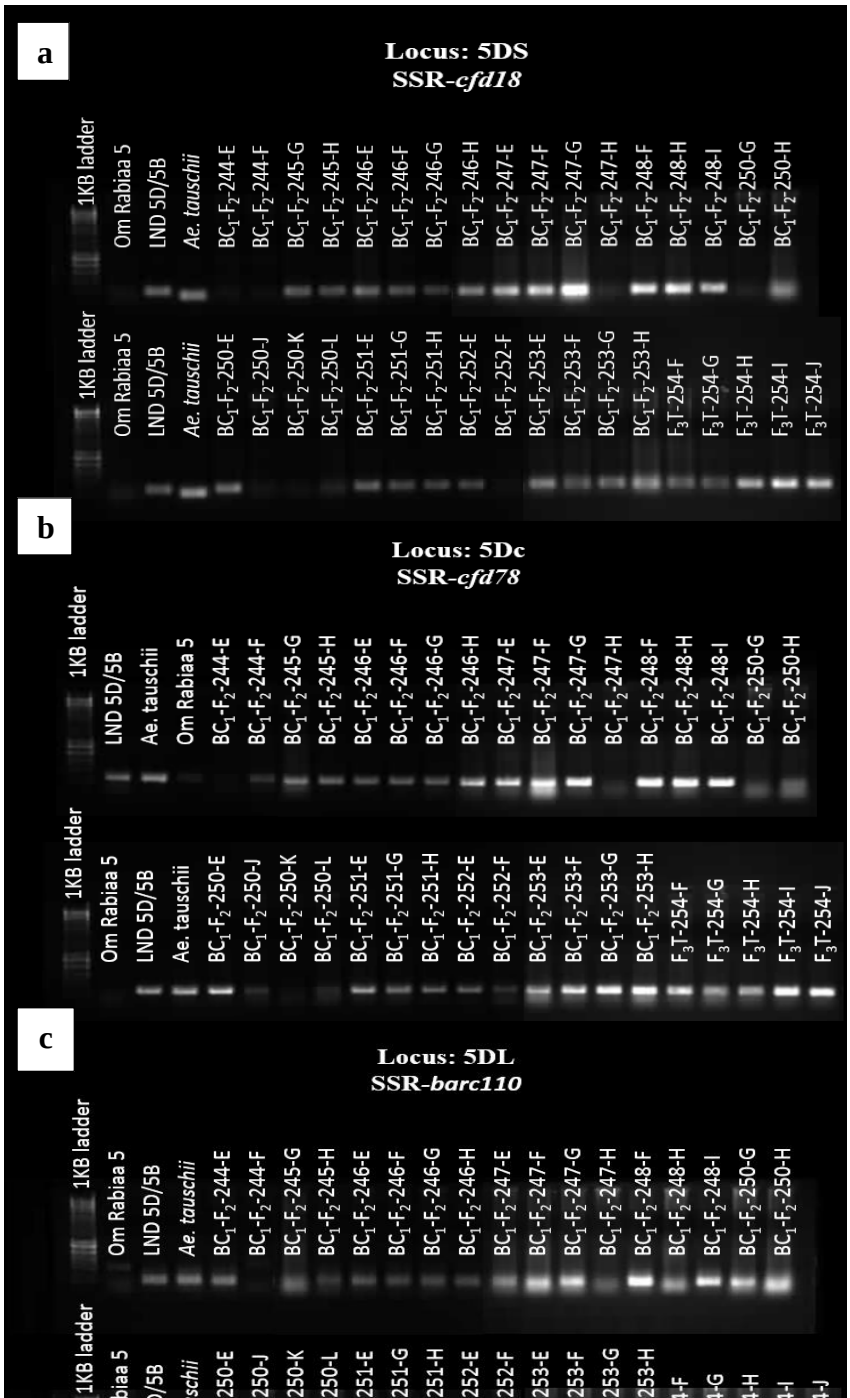


Supplementary material 10. Agarose-gel showing the presence of the 3Dc locus in the BC_1-F_2-250-H, -J and –K (yellow) sister lines using the SSR marker *barc42*.

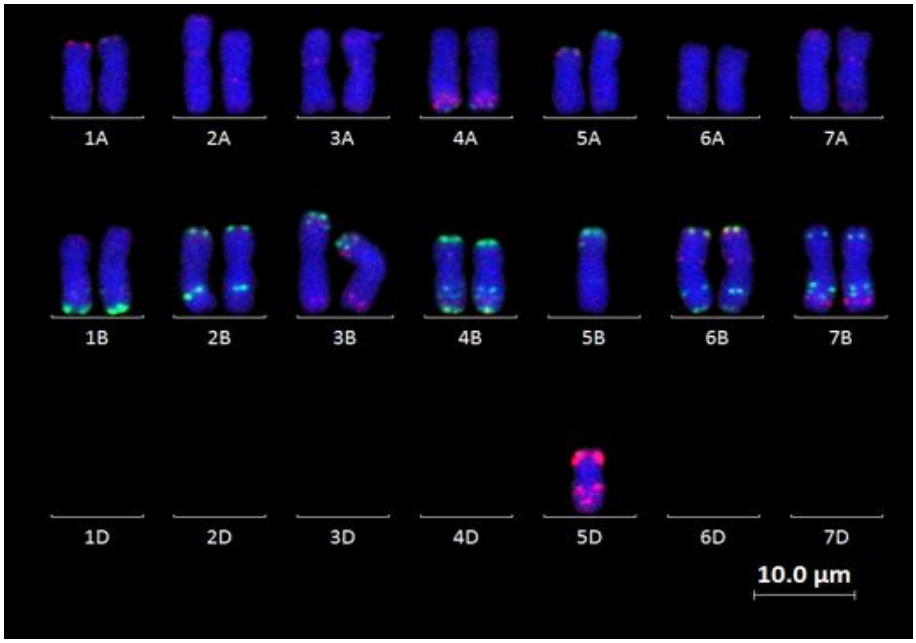
Supplementary material 11. Agarose-gel using the barac334 (a) and cfd284 (b) SSR markers showing the amplification of the 4Dc and 4DL loci, respectively, in the BC_1-F_2-248-F and -I and BC_1-F_2-250-L lines (yellow).



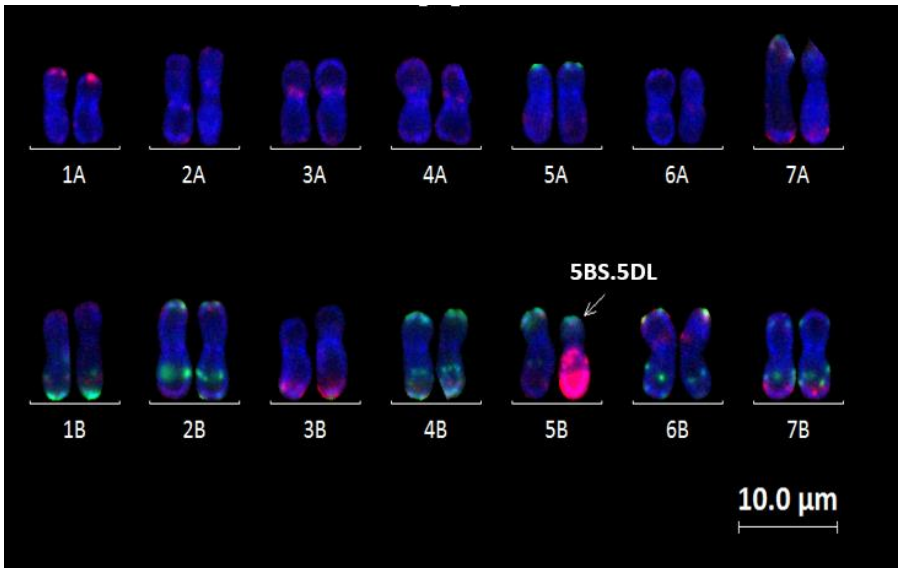
Supplementary material 12. Agarose-gel of BC_1-F_2 and F_3T lines for the 5DS (a), 5Dc (b) and 5DL (b) locus using the 5D specific SSR markers, *cfd18*, *cfd78* and *barac110*, respectively.



Supplementary material 13. FISH-based karyotype of BC_1-F_2-245-G using Oligo-pAs.1 (red) and Oligo-pSc119.2 (green) probes counterstained with DAPI (blue) showing a monosomic 5D (5B) substitution.



Supplementary material 14. FISH based karyotype of the metaphase spread of BC_1-F_2-244-E using Oligo-pAs.1 (red) and Oligo-pSc119.2 (green) FISH-probes counterstained with DAPI (blue) showing 5DL introgression.



Supplementary material 15. Mc-FISH based karyotype of the metaphases spread of the BC_1-F_2-250-H line using the Oligo-pAs.1 (red) and Oligo-pSc119.2 (green) probes constrained with DAPI (blue) showing 3DL-5BL translocation.