Wheat/wild relatives interspecific crosses to identify male fertility restorer factors in alloplasmic lines

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M.Sc.

Thesis submitted to the University of Nottingham for the Degree of Doctor of Philosophy

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January 2019

Abstract

Wheat is an important crop and represents an essential component of the global food security. Hybrid wheat, which currently occupies only a niche sector in commercial wheat production because of several barriers could help facing the challenges of a growing population and climate changes.

This PhD thesis focused particularly on the improvement of the hybrid seeds production based on an efficient cytoplasmic male sterility system (CMS). The first part of the project consisted of the study of wide range of wild relatives (from the *Aegilops*, *Secale*, *Thinopyrum* and *Triticum* genus) as potential source of male fertility restoration genes against *T. timopheevii* cytoplasm. Their genomes were tested through various type of material including amphidiploids and introgression lines. Different strategies were used to reduce the impact of the post-zygotic barriers. Characterization of the fertile hybrid lines was carried out by combining cytogenetics analysis (GISH and FISH) with molecular genotyping (KASP markers). We identified five new restorer chromosomes. The chromosomes 4RL of *S. anatolicum* and 6G of *T. timopheevii* were restoring partially the male fertility in the presence of *T. timopheevii* cytoplasm. High level of restoration was observed in presence of the chromosomes $1A^t$ of *T. timopheevii*, $5S^{sh}$ and $6S^{sh}$ of *Ae. sharonensis*.

The second part of the project focused on the development of a new CMS system based on *Ae. sharonensis* cytoplasm. Wheat male sterile alloplasmic lines were successfully developed. Male fertile alloplasmic lines were produced, indicating the presence of restorer genes into wheat genome against *Ae. sharonensis* cytoplasm. The wide set of wheat / *Ae. sharonensis* addition, substitution and translocation lines produced could be source of new restorer factors.

Acknowledgements

I would like to express my sincere gratitude to my supervisors Dr. Julie King and Prof. Ian King for welcoming me in their team during these four years giving me the opportunity to learn different technics. I would like to thank them particularly for their continuous guidance through the different parts of the project and the sharing of their knowledge which helped me to understand my subject.

I express my gratitude as well to Limagrain for funding this PhD. My sincere thanks go to Alain Murigneux who designed this project and followed the first steps, Jeremy Derory who took over. I really enjoyed all the discussion we went through during our meetings. I express my gratitude to Pierrick Varenne who was present from the start to the end, providing me good advice over the four years and always welcoming me warmly at his breeding station to share his work and ideas. Thanks to all the different members of Limagrain that I met in the different Limagrain centres, who were always happy to spend some time with me, especially my old colleagues of Clermont-Ferrand.

During these four years I met various people who helped me in different ways. I would like to thank all the past and present members of the King's group. Particularly many thanks to Dr. Surbhi Grewal for her help with the KASP markers analysis, Dr. Stella Edwards for general advice, Dr. Cai-yun Yang for sharing her knowledge of cytogenetic techniques, Duncan Scholefield and Stephen Ashling who helped me many times in the glasshouse and advised me on the genotyping protocols and all the other members who were always happy to answer to my questions. A special thanks to Steve for our joyful discussions about Brexit, food and elephants during PCR cycles.

I would like to thank all the people I shared my lunch time during these four years. It was the occasion of lively discussion about worldwide customs and

food sharing. A special thanks to Urmila for sharing the same flat for more than two years. Thank you for all these moments of happiness and laughters. Thanks to Rory who took care of me during the last year, helping me discovered some beautiful English landscapes and cities. I won't forget the cleaning ladies of Plant Science bulding who give me the energy to start my work by their daily warm welcome.

I would not have gone through these four years without the constant support of my family, particularly my parents who supported me over the hardships and who visited me be on a regular basis. I am extremely grateful to my father who is now an expert on food shipment! Thank for trying so hard to understand the subject of my PhD, for the hours spent on the seeds threshing and the excellent advice on how to improve my presentations. I won't forget my late grandparents, specially Bon Papa who transmitted to me the virus of plants and the scientific rigor.

Thank you to all my friends in France who stayed in touch despite of the distance and were always happy to give me some time when I came back home. I would particularly thank my friends who crossed the Channel to visit me with some cheese in their suitcase. A special thanks to Pauline who daily supported and advised me during the last four years, always happy to host my empty stomach for a week-end and to share her extraordinary adventures in Ardèche, Alsace and now California. Finally, I would like to thank Guillaume who helped me so much by his patience, his kindness and his constant support.

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List of abbreviations

A-line: Male sterile line
Ae.: *Aegilops*
AFLP: Amplified Fragment Length Polymorphism
Am.: *Ambylopyrum*
ATP: Adenosine triphosphate
BC: Back cross
BDM: Bateson-Bodzhansky-Muller
B-line: Maintainer line
bp :Base pair
cdk: Cyclin dependent kinase
CHA: Chemical Hybridizing Agent
Chr. : Chromosome
cm: centimeter
CMS: Cytoplasmic male sterility
CO: Crossover
CS: Chinese Spring
Da: Dalton
DArT: Diversity Array Technology
DNA: Deoxyribonucleic acid
dNTP: Deoxyribonucleotide triphosphate
dUTP: Deoxyuridine Triphosphate
e.g. : *exempli gratia*
EGMS: Environment genic male sterility
EMS: Ethylmethane sulphonate
F_1 : Filial 1
FDR : First division restitution
FISH : Fluorescent *in situ* hybridization
Gc : Gametocidal
GCA : General combining ability
gDNA: Genomic DNA
GBS: Genotyping by Sequencing
GISH: Genomic *in situ* hybridization
GM : Genic male sterility
GP: Gene pool
Ha : Hectare
hQTL : Heterosis quantitative trait loci
Hz : Hertz
i.e: *id est*

Igc : Gametocidal inhibitor
k: Kilogram
KASP: Kompetitive allele specific PCR
Kr : Crossability gene
L : Long
L. : Linné
Lr : Leaf rust
m : Million
M: Molair
ME : Modification enzyme
mL: Millilitre
MLH1 : Double Hollyday Junction Marked
Ms : Male sterile gene
Mt : Million tonnes
mt: Mitochondrial
Ne: Necrosis gene
NGS: Next Generation Sequencing
NMS: Nuclear encoded sterility
No.: Number
ORF : Open reading frame
PCD : Programmed Cell Death
PCMS : Photoperiod-sensitive cytoplasmic male sterile
PCR : Polymerase chain reaction
PGI: Pollen germination inhibitor
PGMS: Photoperiod-sensitive genic male fertility
Ph: Pairing homoeologous
PLUG: PCR based landmark unique gene
Pm: Powdery mildew
PPR: Pentatricopeptide repeat
PTGMS: Photoperiod-sensitive and temperature-sensitive genic male fertility
QTL : Quantitative trait loci
R: Resistance
RAPD : Random Amplified Polymorphic DNA
RE: Restriction enzyme
Rf : Restorer of fertility
RFLP: Restriction Fragment Length Polymorphisms
Rht: Reduced height loci
R-line: Restorer line
ROS: Reactive oxygen species
RNA: Ribonucleic acid
rpm: Rotation per minute

S : Short
S.: *Secale*
SC: Seed colour marker gene
SC: Synaptonemal complex
SCA: Specific combining ability
SDR: Second division restitution
SDMG: Single division meiosis
SI: Self-incompatibility
SNP: Single Nucleotide Polymorphism
SPT: Seed Production technology
SSC: Saline Sodium Citrate
ssp.: Subspecies
SSR: Simple sequence repeats
Sr: Stem rust
T. : *Triticum*
TE: Tri EDTA
Th.: *Thinopyrum*
TGMS: Temperature-sensitive genic male fertility
µg: microgram
µL: microliter

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I. Chapter 1: General Introduction

I.1. Introduction

Wheat is one of the major crops cultivated around the world. In 2016, 749 m tonnes were produced, corresponding to 220 m ha cultivated (http://faostat.fao.org/). The success of this crop is based on its ability to grow in a wide range of environments (Feldman 1995). Within the wheat family, *Triticum aestivum* or bread wheat is the most cultivated (around 95% of the production). The other important species cultivated is *T. durum*, or pasta wheat, which is more adapted to dry areas (Shewry, 2009). Wheat has an important contribution in the human diet as it provides 20% of the daily calories (Braun et al., 2010). It is also a source of proteins, fibre, vitamins and minerals (Shewry and Hey, 2015). According to the projection of several studies, the world population will reach 9.3 billion by 2050 ("World Population United Nations" 2010.). Moreover, consumption habits of the population have changed, involving the consumption of wheat products in countries where wheat cannot be grown (Curtis and Halford, 2014; Shewry and Hey, 2015). In order to meet the demand in the coming decades, wheat yield has to increase by 1.7% per year until 2050 (Rosegrant and Agcaoili, 2010), but the current genetic gain is around 1% per annum (Dixon et al., 2009). In some cases it has been shown that yield is stagnating (Brisson et al., 2010; Charmet, 2011; Godfray et al., 2010; Ray et al., 2013). The genetic gain is partially counteracted by climate change. The challenge becomes growing more food on an equal or reduce arable area, with less input (water, pesticides and fertilizers) (Reynolds et al., 2012). The most sustainable solution to increase yield rate is to improve genetic potential. Exploitation of heterosis, or hybrid vigour, looks to be a promising solution for yield improvement in wheat (Singh et al., 2015; Whitford et al., 2013). Hybrid varieties have been developed for many crops such as maize, oilseed rape or sunflower. Hybrid wheat breeding currently represents a minor part of production because the complexity of the hybrid system counters the benefits.

I.2. What is wheat?

I.2.1. Taxonomy of the wheat group

Common wheat, *T. aestivum*, belongs to the grass tribe Triticeae in the grass family *Poaceae* (*Gramineae*). The tribe Triticeae was divided in to 20 – 30 genera which regrouped about 360 species (Bernhardt, 2015). The Triticeae tribe includes the genera *Hordeum* (barley) and *Secale* (rye) or *Aegilops* as well as the genus *Triticum*. The basic haploid chromosome number of the Triticeae tribe is 1x=7 (Sears, 1954).

Classification of the wheat group has been the subject of several studies (Barkworth and Bothmer, 2009). Some authors restricted it only to the genus *Triticum* (species having the A genome alone or in combination, for example AB or ABD). According to other authors, however, the wheat group also includes the *Aegilops* and *Amblyopyrum* genera (Eig, 1929; Hammer, 1980; van Slageren, 1994). The complexity of the phylogeny of the *Triticum* – *Aegilops* complex can be explained by its young age and by the fact that multiple hybridizations have occurred between species belonging to different genera of the Triticeae (Feldman and Levy, 2015).

In 1918 Sakamura classified cultivated wheat species into three different groups: diploid wheat (2n = 2x = 14), tetraploid wheat (2n = 4x = 28) and hexaploid wheat (2n = 6x = 42). The wheat group contained 13 diploid species (van Slageren, 1994) (**Table I-1**). These species contained eight distinct genomes: A (A, A^b or A^m), C, D, M, N, S (S, S^s, S^b, S^l or S^{sh}), T and U (Feldman and Levy, 2015). The wheat group also included 18 different allopolyploid species (12 tetraploids and 6 hexaploids) which were classified into three major genome groups A, U and D based on the pivotal or unaltered genome present (Zohary and Feldman, 1962). These polyploid species are the results of inter-specific or inter-generic hybridization followed by chromosome doubling (Kihara, 1924, 1919; Percival, 1921; Sax, 1927, 1921). The species differed from each other by accumulation of mutations in the unshared genomes (Mirzaghaderi and Mason, 2017; Zohary and Feldman, 1962).

Table I-1 Species belonging to the genus *Triticum* and *Aegilops* and their genomic composition

Ploidy level	Genus	Species	Genome
Diploid	*Amblyopyrum*	*muticum*	T
	Aegilops	*speltoides*	S
		searsii	S^s
		bicornis	S^b
		longissima	S^l
		sharonensis	S^{sh}
		tauschii	D
		caudata	C
		comosa	M
		uniaristata	N
		umbellulata	U
	Triticum	*monococcum*	A^m
		urartu	A
Tetraploid	*Aegilops*	*biuncialis*	UM
		geniculata (ovata)	MU
		neglecta (triaristata)	UM
		columnaris	UM
		triuncalis	UC, CU
		kotschyi	SU
		peregrina (variabilis)	SU
		cylindrica	CD
		crassa	DM
		ventricosa	DN
	Triticum	*turgidum*	BA
		timopheevii	GA^t
Hexaploid	*Aegilops*	*recta (triaristata)*	UMN
		vavilovi	DMS
		crassa	DDM
		juvenalis	DMU
	Triticum	*aestivum*	BAD
		zhukovskiy	GAA^m

Classification of the *Triticum* group itself has caused many discussions. Two main classifications are used. The first, mostly used in Western countries, was established by Mac Key (1966, 1977) and revised by van Slageren (1994). This classification was based on a small number of morphological traits and recognize six different species. The second classification, mostly used in Eastern countries, was by Dorofeev et al. (1997) and revised by Goncharov

(2002). This classification used a very fine discrimination of morphological traits and recognizes 29 different *Triticum* species. The classification of van Slageren (1994) will be followed in the rest of the work.

The *Triticum* genus is composed of six different species and several subspecies (van Slageren, 1994). The two diploid species are *T. urartu* (AA genome), which exists only in its wild form, and *T. monococcum* L. (A^mA^m) which includes *T. monococcum* ssp. *aegilopoides* (synonym *T. boeoticum*) as the wild form and the cultivated form *T. monococcum* ssp. *monococcum*. The two tetraploid species are *T. timopheevii* (A^tA^tGG) including the wild subspecies *T. timopheevii* ssp. *armeniacum* (synonym *T. araraticum*) and the cultivated form *T. timopheevii* ssp. *timopheevii* and *T. turgidum* L. (AABB), which includes the wild subspecies *T. dicoccoides* (wild emmer) and the cultivated subspecies *T. turgidum* ssp. *dicoccon* (cultivated emmer), ssp. *durum* (durum or macaroni wheat), ssp. *polonicum* (Polish wheat), ssp. *turanicum* (Khorassan wheat), ssp. *turgidum* (Rivet wheat), ssp. *carthlicum* (Persian wheat) and ssp. *paleocolchicum* (Georgian wheat). The two hexaploid species are *T. zhukovskyi* (A^mA^mAAGG) and *T. aestivum* (AABBDD), divided into 5 subspecies: ssp. *aestivum* (common wheat), ssp. *compactum* (club wheat), ssp. *sphaerococcum* (Indian dwarf wheat), ssp. *macha* and ssp. *spelta* (spelt).

I.2.2. Origin of wheat

Bread wheat is a hexaploid (2n = 6x = 42, AABBDD) which resulted from two evolutionary events (**Figure I-1**). The first one took place 50,000 years ago and was the neotetraploidization between a diploid close to *T. urartu* (donor of the A genome) with a species from the *Sitopsis* section (donor of the B genome) (Feldman and Levy, 2005). The result of this first hybridization was a tetraploid *T. turgidum* (2n = 4x = 28, AABB) or emmer wheat, to which *T. durum* is closely related (Luo et al., 2007). The second evolutionary event was the neohexaploidization of *T. turgidum* with *Ae. tauschii* (donor of the D genome), which led to *T. aestivum*, 8,000 years ago (Feldman, 2001).

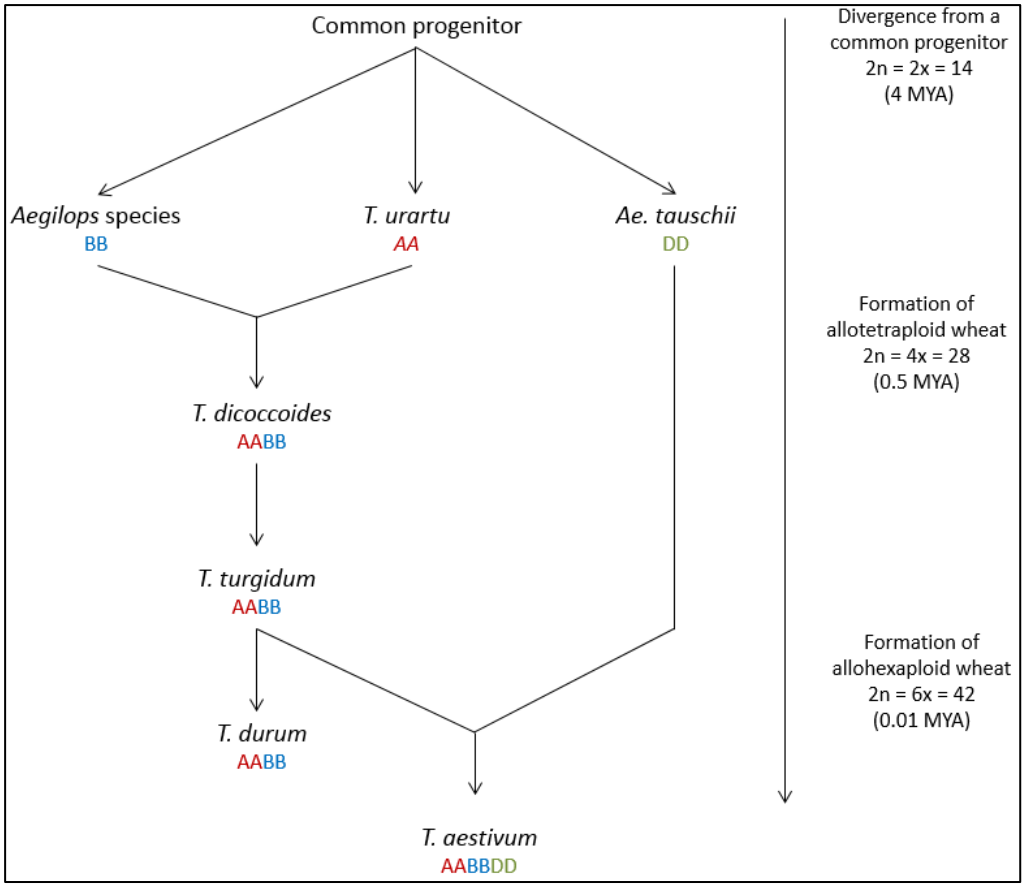


Figure I-1 The evolution of common wheat
The genomic composition is indicated below the name of the species

While it has been established that *T. urartu* was the donor of the A genome (Dvořák et al., 1993) and *Ae. tauschii* the donor of D genome (Dvořák et al., 1998a; Kihara, 1944; McFadden and Sears, 1944, 1946), the origin of the B genome is still the subject of discussion. Several species have been proposed to be the donor of the B genome: *T. villosum* (Montebove et al., 1987), *T. urartu* (Johnson, 1975; Johnson and Dhaliwal, 1976), *Ae. bicornis* (Sears, 1956a), *Ae. longissima* (Ogihara and Tsunewaki, 1982), *Ae. searsii* (Kihara, 1954), *Ae. sharonensis* (Kushnir and Halloran, 1981). The last potential donor of the B genome proposed was *Ae. speltoides* (genome SS), considered as the closest ancestor (Riley et al., 1958; Zohary and Feldman, 1962), given to the B genome a monophyletic origin (derived from a single ancestor). Molecular studies showed that *Ae. speltoides* was the donor of the cytoplasm of common wheat (Dvořák and Zhang, 1990; Haider, 2012; Kilian et al., 2006). Nevertheless, the origin of the B genome is still discussed, some authors

thinking that the species has not been discovered yet, others that the species disappeared or that the B genome has evolved so much compared to the donor. Kilian et al 2007 proposed that the B genome is the result of introgression between several lines of *Ae. speltoides*, which is relevant with the outcrossing nature of *Ae. speltoides*. However, it has been shown that *Ae. speltoides* has diverged from the B genome and thus might not be the ancestral donor (Huang and et al., 2002; Salse et al., 2008). Zhang et al (2017) demonstrated that *Ae. speltoides* was involved in the formation of the B genome but was not the exclusive donor. The B genome could be the result of homoeologous recombination between different genomes of *Sitopsis* section, giving to this genome a polyphyletic origin (derived from more than one ancestor).

The B genome shows a higher genetic diversity than the A and D genome (Petersen et al., 2006). Different hypotheses about the B genome origins were proposed to explain the accumulation of mutations in the B subgenome. This difference could be partially explained by the origin itself of the B genome, coming from the most ancient ancestor (monophyletic origin) or resulting from several recombination of Sitopsis species (polyphyletic origin). Nevertheless, El Baidouri et al. 2017 showed that accumulation of rearrangements in the B subgenome was more important than in the other subgenomes after the two hybridization events. This asymmetric evolution between the three genomes resulted in subgenome dominance (Pont et al., 2013). A dominant subgenome is stable and associated with low duplicated gene loss, whereas a sensitive subgenome is associated with a high duplicated gene loss and increased gene diversity. After the first hybridization event, the A subgenome acted as a dominant genome and the B as a sensitive genome. After the second hybridization, D genome became a dominant genome whereas the AB subgenome acted as a sensitive genome. The D subgenome was the pivotal genome (El Baidouri et al., 2017; Pont et al., 2013). In 2012, Feldman et al., showed that the A genome was controlling morphological traits whereas the B

genome was controlling more of the reactions to biotic and abiotic stress, confirming the hypothesis of the subdominance.

The origin of the D genome has been the subject of discussion as well. The D genome resulted from a homoploid hybridization (hybridization without change of ploidy level leading to a stable, fertile and reproductively hybrid) between the A and B genomes 5 million years ago (Marcussen et al., 2014; Sandve et al., 2015). Indeed, the A and B genomes are more similar to the D genome than they are to each other. Nevertheless, the origin of the D genome seemed to be more complex. Chloroplast studies of 20 species of the *Aegilops* - *Triticum* complex showed that *Ae. tauschii* did not share the chloroplast genome either with *Ae. speltoides* or with *T. urartu* (Li et al., 2015b, 2015c). The authors suggested a nested origin of the D genome. Before or after the homoploid hybridization between A and B genome, a third hybridization occurred with a species belonging to the *Sitopsis* section. This second hypothesis was confirmed by El Baidouri et al., 2017, showing that the third ancestor was involved in at least 19% of the modern D subgenome.

I.2.3. Wheat domestication

Wheat was domesticated 12,000 years ago during the Neolithic time in the Fertile Crescent when humans left the hunting way of life for a settled agriculture (Kilian et al., 2010). During the domestication process, the cultivated form differed from the wild species by traits, referred to as the "domestication syndrome" (Hammer, 1984; Pozzi and Salamini, 2007). The two most important traits modified during domestication were free-threshing and brittle rachis. Other traits were also modified during this process such as increase in seed size, reduction in plant height, reduction of tillering, reduced seed dormancy, vernalisation requirements and heading date (Kilian et al., 2009).

Free-threshing is the loss of tough glumes adhering tightly to the grain. This trait would have been selected by farmers to make the harvest easier and

more efficient. The free-threshing character has been considered to be controlled by minor and major mutations (Jantasuriyarat et al., 2004). The major one was identified as the *Q* locus, located on chromosome 5AL (Endo and Gill, 1996). This locus has pleiotropic effects and influences many other domesticated traits such as spike density (or square headed) (Unrau et al., 1950), softer glumes (Muramatsu, 1963, 1986), tougher rachis (Jantasuriyarat et al., 2004) and smaller plant (Kato et al., 1999, 2003; Muramatsu, 1963). The wild relatives and primitive wheat carry the *q* allele, or speltoid type, characterized by tenacious glumes and elongated rachis. Cultivated wheat, *T. turgidum* and *T. aestivum* have the *QQ* genotype and are free-threshing. *Q* is considered as an incomplete dominant allele as bread wheat lines having a genotype *Qq* show a spike morphology intermediate between speltoid and square head. Muramatsu (1963) studied the phenotype of substitutions and aneuploid line of chromosome 5A of *T. aestivum* ssp. *spelta* into Chinese Spring background. Below four doses, q allele bellows as a null allele and leads to a speltoid phenotype. Five doses of q allele (qqqqq) give a squarehead that corresponds to the phenotype obtained with two doses of the Q allele (QQ). Muramatsu (1963) concluded that *q* was hypomorphic to *Q* (i.e. the mutation leads to a reduction of the gene function) and suggested that a 2.5 dose of *q* is equal to 1 dose of *Q*. He also emphasized the importance of the genetic background. The mutation which led from the *q* to the *Q* allele occurred only once, and thus tetraploid and hexaploid wheat thus carry the same *Q* allele (Simons et al., 2006). The *Q* gene has been cloned and its sequence shown to be similar to the Arabidopsis *APETALA2* (*AP2*) gene (Faris and Gill, 2002; Faris et al., 2003). In 2006, Simons et al., validated this result by identifying the *AP2-like* (*WAP2*) gene as *Q*.

A second major gene is involved in the control of free threshing traits. In 1969, Kerber and Dyck noticed that some synthetic hexaploid wheats, with the *QQ* genotype, were not free-threshing. The suppression of the free-threshing character was due to the presence of a partially dominant gene *Tg* (tenacious glumes). The *Tg* gene inhibits the expression of the *Q* gene. This gene derived

from *Ae. tauschii* and is located on chromosome 2DS (Nalam et al., 2007). A free-threshing hexaploid wheat has the genotype *tgtg*, *QQ* (Kerber and Rowland, 1974).

The second most important trait linked to wheat domestication is the modification of the brittle rachis. When spikes of wild emmer [*Triticum turgidum* L. ssp. *dicoccoides* (Korn. ex Asch. and Graebner) Thell.] reach maturity, their brittle rachis disarticulate spontaneously and fall on the soil, assuring the seed dispersion (Kilian et al., 2009). Through domestication, humans have selected plants with a tough rachis to avoid the dispersion of the spikelet. A single dominant gene Br_1 (Brittle rachis), linked to rachis fragility, has been located on chromosome 3DS in a Tibetan common wheat (Cao et al., 1997; Chen et al., 1998). Two other dominant genes have been discovered, Br_2 on 3A and Br_3 on 3B (Nalam et al., 2006).

I.3. State of art of hybrid wheat

I.3.1. Heterosis: definition and genetic basis

After a first description by Darwin in 1876, heterosis was rediscovered in maize at the beginning of the twentieth century and has been defined as the superiority of heterozygous genotypes over their corresponding parental genotypes (Shull, 1908). The commercial exploitation of hybrid maize started in the 1930s (Duvick, 2001). Heterosis can bring a yield gain between 15% and 50% depending on the crop (Lippman and Zamir, 2007). Whereas heterosis is widely used for crop improvement, the mechanisms behind it are still poorly understood as heterosis is a complex phenomenon resulting from several mechanisms.

Different hypotheses have been proposed to explain heterosis, but it seems that this phenomenon is the result of the combination of several genetic effects (Birchler et al., 2003; Fu et al., 2015; Schnable and Springer, 2013).

- The first hypothesis is the dominance hypothesis (or complementation) based on the introduction by one parent of desirable alleles repressing the

deleterious effect of alleles from the other parent. Parents have to carry deleterious alleles at different loci. Several criticisms have been raised against this dominance hypothesis. Indeed the inbred line produced from a hybrid carrying only favourable alleles should have the same performance as the hybrid, which is not the case (Birchler et al., 2003; Schnable and Springer, 2013). Another point is that according to this model, the heterosis gain should decrease over time as the breeders produce inbred parental lines with less and less deleterious effects. But the magnitude of heterosis has actually increased over the decades (Birchler et al., 2003). Another criticism is that all the recessive alleles are not deleterious and have to be maintained in the parental lines for agronomic reasons (Fu et al., 2015).

- The second hypothesis is the overdominance hypothesis (or complementary interaction) which attributes heterosis to the superior fitness of heterozygous genotypes over homozygous genotypes at a single locus. This model does not take into account dominance – recessive relationships. Moreover, heterozygosity at some loci can lead to unviability (Fu et al., 2015). The pseudo dominance hypothesis is an intermediate between the dominance and overdominance hypotheses if two dominant alleles are tightly linked in repulsion (two genes are in repulsion phase when each homologous chromosome has one dominant and one recessive allele from the two genes). This situation can complicate QTL analysis of the heterosis (Fu et al., 2015; Schnable and Springer, 2013).

- The two first hypotheses are based on a single locus theory. The last hypothesis is the epistasis between the non-allelic genes (Fu et al., 2015). In rice, epistasis plays an important role in the heterosis of yield (Yu et al., 1997) and in the control of heading date and plant height (Yu et al., 2002).

It has been noticed that inbreeding depression (ie. reduction or loss in vigour and fertility through a selfing process, caused by the accumulation of deleterious recessive alleles) occurs faster in the tetraploids than in the diploids despite the fact that the number of completely homozygous lines after selfing is fewer in the tetraploid compared to the diploid (Birchler et al., 2003).

This inbreeding depression in polyploids might be caused by the allelic dosage effect. This is another proof that the complementation is not adequate. Heterosis seems to be a combination of these three hypotheses (Schnable and Springer, 2013). For example in rice, plant height results from the combination of dominance and epistasis (Shen et al., 2014). The important question is whether theses hypotheses are enough to explain heterosis or whether additional mechanisms are needed.

The genetic basis of heterosis has been studied by the mapping of heterosis quantitative traits (hQTL). Nevertheless the choice of the model, the experimental design and the statistical power influences the interpretation of the results leading to a dominance or overdominance conclusion (Lippman and Zamir, 2007; Reif et al., 2005). Fine-mapping and positional cloning are then required to identify and characterize the underlying genes involved in this phenomenon. Many examples of heterosis have a multigenic nature. Many hQTL have been detected for a variety of traits in several species such as vegetative growth, flowering time, flowers per inflorescence or grain weight (Schnable and Springer, 2013). However, in some cases only a single gene is involved. For example one gene has been found to be responsible for hybrid vigour in maize (Dollinger, 1985), another one the *SINGLE FLOWER TRUSS* gene is responsible for an increase in yield of up to 60% in tomato (Krieger et al., 2010).

At the organelle level, hybrids show differences compared with their parents. Increase of the synthesis of ATP in the mitochondria, increase of the photosynthesis activity in the chloroplasts and increase of the concentration of gibberellin (the growth hormone) have been reported (Reif et al., 2005). Hybrids also show a better energy use efficiency (Fu et al., 2015).

Finally, epigenetic modifications are associated with heterosis. Many studies have shown DNA methylation, histone modification or regulation by small RNAs effect heterosis (Fu et al., 2015; Schnable and Springer, 2013). For

example it has been reported that inbreeding causes an increase in DNA methylation and that hybrids show less methylation than their parents (Reif et al., 2005). Amount of small RNAs is decreases in the hybrid compared to the inbred parents (Fu et al., 2015). Nevertheless, it remains unclear if DNA methylation and small RNAs are the cause or the consequence of heterosis.

I.3.2. Heterosis in wheat

Heterosis in wheat was first reported for plant height in 1919 by Freeman. In 1963, Birggle showed hybrid vigour for yield, ranging from 0 to 100%. According to Hoisington et al., (1999), wheat expressed a level of heterosis between 10 to 25%. However, most of the studies were done on only a few individuals and were tested in few environments (Virmani and Edwards, 1983). In 2012, Longin et al., summarized 20 years of studies involving at least three different environments. They showed an average of yield heterosis from 3.5 to 15%. Moreover hybrids showed a higher yield stability than the inbred lines and yield gain was particularly important in extreme environmental conditions (Mühleisen et al., 2014). Hybrids did not show heterosis only for yield but also for biotic and abiotic stress. Susceptibility of the hybrid compared to the parents was reduced in the case of frost (-7.2%), leaf rust (-8.4%) (Longin et al., 2013), Septoria tritici blotch (-9.3%) (Longin et al., 2013; Miedaner et al., 2013) and Fusarium Head Blight (-9%) (Miedaner et al., 2017).

The main yield components in wheat are the number of spikes per square meter, the grain number per spike (the number of spikelet per spike and the spikelet fertility) and a 1000 grains weight (Virmani and Edwards, 1983). Nevertheless, heterosis results from the combination of these components and a single component can be the unique source of heterosis. Compensation for one component by the others has been shown (Pinthus and Millet, 1978). Prediction of such a complex trait as yield controlled by several loci with small effect, can be done by genomic selection.

Hybrid seed production seems to be the future of plant breeding not only because of the yield gain but also because of the security on the return on investment for the breeding companies due to the existence of depression in the selfing generation preventing the multiplication of the seeds by the farmers.

I.3.3. Commercial hybrid wheat production requirements

Despite these advantages, less than 1% of the wheat cultivated around the world is hybrid (Longin et al., 2012) contrary to maize hybrids which represent more than 65% (Fu et al., 2014a). The requirements for a successful hybrid wheat breeding program are good levels of heterosis, a cost-effective system to produce hybrid seed, and a competitive selection gain of hybrid versus line breeding (Longin et al., 2012). Selection gain is defined as $\Delta G = (ih\sigma_G)/y$, where i is the selection intensity, h the square root of the heritability, σ_G the square root of the genetic variance and y the number of years required to finish one breeding cycle (Cochran, 1951). In order to counterbalance the seed cost production in a hybrid system, a yield advantage of 1 Mt/ha for hybrids compared to inbred lines is needed (Weissmann and Weissmann, 2002). A surplus of 1.86 Mt/ ha has been found in the best hybrids compared to the elite inbred lines showing that hybrids are potentially competitive to inbred lines (Gowda et al., 2012). The two crucial points which have to be improved to build the competitiveness of the hybrids compared to the inbred lines are the development of distinct genetic groups within the parental lines (Longin et al., 2012, 2014a) and the redesign of the floral morphology to ensure cross pollination (Whitford et al., 2013). These two improvements are resource and time consuming, threatening the selection gain of the hybrid versus the inbred line. Indeed length of the selection cycle is a crucial parameter in the calculation (Longin et al., 2012).

I.3.3.1. *Creation of a heterotic group*

The first step in hybrid production is the choice of the parents as heterosis increases with the genetic distance between parents (East, 1936). Potential

parents have to be ordered into genetically divergent groups to maximise the heterosis (Reif et al., 2005). These form the heterotic groups while a heterotic pattern is a specific pair of heterotic groups expressing high heterosis (Melchinger and Gumber, 1998).

The lower heterosis observed in autogamous compared to allogamous crops can be explained by the low diversity existing between parental lines (Falconer and Mackay, 1995). Indeed, wheat breeding strategies led to the production of elite lines after intensive exchange of material across countries. The result is the absence of genetically distinct groups in wheat (Boeven et al., 2016a; Whitford et al., 2013). The challenge is therefore the establishment of heterotic groups in order to maximise heterosis (Longin et al., 2012, 2014a). Boeven et al., (2016), studied the genetic diversity of a global collection of 1,110 winter wheat varieties with a focus on the European cultivars. Despite the absence of major population structure, a distinction existed between the European and Asian cultivars, and within the European group the eastern germplasm was different to the western germplasm. The easiest solution to increase diversity between parents is to use lines coming from different environments (Koekemoer et al., 2001). However, this brings problems of vernalisation, photoperiod sensitivity and frost tolerance (Longin et al., 2012). Strong differences in flowering time are particularly problematic. For example, Asiatic cultivars tend to be one or two weeks earlier compared to European cultivars, considered as the male pool, leading to the impossibility of crossing these parents in the field (Boeven et al., 2016a).

In order to establish, maintain and then improve heterotic groups in wheat, a united framework, *HyBFame*, has been proposed (Boeven et al., 2016a). The first step consists in the choice of the parents within the high yield elite lines, a choice aided by molecular marker analysis to ensure genetic diversity. The choice of these lines also relies on their ability to ensure cross pollination. Through factorial crosses the lines showing the higher GCA (General Combining Ability) are selected. Reciprocal recurrent selection is settled down

in order to improve the GCA, resulting in an increase of the genetic distance between the two pools. New material can be introduced in a pool to increase the intra group genetic diversity (floral morphology or adaptation traits for example). Introgression of small interesting fragments by back crosses is the best solution not to disrupt the heterotic pattern.

I.3.3.2. *Optimization of the breeding program*

Because of the high number of potential parental lines, field evaluation of hybrid combinations is not feasible. Reliable hybrid performance prediction is very thus important (Longin et al., 2013). The parental lines are selected for their performance *per se* or for their GCA by a cross with a tester belonging to another heterotic group (Reif et al., 2005). Indeed prediction of hybrid performance by using GCA shows high accuracy when the variance due to the GCA is higher than the variance due to the specific combining ability (SCA) (Longin et al., 2013). It has been shown that the establishment of heterotic group leads to a decrease of $\sigma_{SCA}^2/ \sigma_{GCA}^2$ and allows early testing to be more effective (Longin et al., 2012; Reif et al., 2005, 2007). The SCA can also be reduced by the used of multiple tester (Longin et al., 2014b).

Genomic selection can be used to predict complex traits influenced by many loci. This method is based on genome-wide markers to predict the breeding value of individuals (Whitford et al., 2013). Hybrid wheat performance with genomic selection has been studied with a focus on yield (Zhao et al., 2013), septoria tritici blotch (Miedaner et al., 2013), heading time and plant height, which are two important traits for hybrid wheat production (Zhao et al., 2014). Nevertheless, more studies are needed to select the best model. Longin et al., (2015), proposed one stage genomic selection followed by one-stage phenotypic selection as the best breeding strategy.

I.3.3.3. *Improvement of the floral morphology to enhance cross pollination*

Because of the autogamous nature of wheat, modification of the reproductive system is needed to produce hybrid seed. The quasi totality of the seeds are the result of self-pollination, as out-crossing is estimated to be around 6% (Hucl, 1996; Martin, 1990). Understanding of the floral architecture is the basis to redesign the wheat flower according to the criteria needed for hybrid production. The wheat inflorescence is an ear or spike, which is a composed of spikelets attached on a main axis, the rachis. Each spikelet is made up of two bottom glumes (glumea) and 2 to 5 florets. A floret is made of two glumes, the lemma and the palea, which envelope the male and female reproductive organs. The lemma can bear an awn. As a wheat flower is hermaphrodite, both female and male organs are present in a floret. The male organ consists of 3 stamen composed of a filament and an anther containing the pollen. The female organ, the pistil, is composed of an ovary bearing two fluffy stigma. At anthesis time, the lodicule (a small organ located at the base of the floret) becomes turgid and swollen, resulting in the opening of the lemma and palea. The filaments of the stamen elongate, pushing the anthers out of the floret. At the same time, the stigma curve away from each other and spread out. Anther dehiscence results in the liberation of the pollen which falls on the feathery stigma. After some time the lodicule loses its turgidity and collapses, resulting in the closing of the floret at which point flowering has been completed (Vries, 1971; Whitford et al., 2013). Nevertheless a 'second opening' can occur a few days after the 'first opening' which has been described above (Pickett, 1993). In most cases this happens when the pollination did not occur at the first opening. Okada et al., (2018) showed that this 'second opening' is characterized by an increase in the radial size of the ovary pushing away the palea and the lemma. This increase in the size of the ovary is caused by the absence of the degradation of the mesocarpe and endocarp layers of the ovary. Low expression of *TaVPE4* results in lower expression of a vacuolar processing enzyme (VPE) which would normally play a role in the programmed

cell death of the ovary. This 'second opening' provides the opportunity for the florets to change from self-pollination to open pollination (Okada et al., 2018).

In regards to the floral morphology of wheat, several traits need to be improved to achieve cross pollination: opening of the glumes, soft lemma, large lodicule, size of the stigma and anther extrusion, quantity of pollen produced, duration of flowering (Vries, 1971). These traits are highly genotype dependant, meaning that breeding can highly improve the parental lines (Singh et al., 2010). For example, some modern wheat varieties show a higher degree of spikelet opening leading to a higher outcrossing rate (Hucl, 1996; Vries, 1971). The ideotype male line would show good extrusion of the anthers containing a huge amount of long-life pollen over an extended period of time. The pollen should have good aerodynamic qualities as well. The ideotype female line would have the following characteristics: male sterile, long stigmatic hairs, well extruded and a long-time of flower opening in order to catch as much pollen as possible (Pickett, 1993; Vries, 1972; Whitford et al., 2013). Moreover flowering time between the two parents has to be synchronised (Virmani and Edwards, 1983). Hence pollen quantity, spread and viability appears to be a major problem in hybrid wheat production (Longin et al., 2012). Pollen life is less than one hour (D'SouzA, 1970; Fritz and Lukaszewski, 1989). On average, florets open during 3-4 days (Vries, 1971). However, it is interesting to notice than florets of male sterile plants opened later and remained open until 9 days after complete heading (Okada et al., 2018).

In addition to the cleistogamic character of wheat flower, several traits have been selected during evolution and domestication to reduce flower opening, e.g., traits such as stiff and large glumes, stiff lemma and small anthers (Whitford et al., 2013). The *Q* gene, a major factor in the domestication of wheat, has an important role in floral morphology through inducing free threshing and compact spike character (Simons et al., 2006). Squared head spikes show opening of the lower glumes, a trait required for inbred line

production (Vries, 1971). Another important trait is the height difference between the two parents. In order to maximise pollination, the male line has to be taller than the female (Virmani and Edwards, 1983). Major dwarfing genes, *Reduced height loci* (*Rht*), particularly the two homoeoloci *Rht-B1b* and *Rht-D1b* were wide spread through wheat cultivars during the green revolution (Hedden, 2003; Wilhelm et al., 2013). These homoeoloci are located on group 4 chromosomes of wheat (Achard, 2006). A new gene *Rht24* has been identified on chromosome 6A with comparable height effect (Würschum et al., 2015, 2017a, 2018). *Rht-B1b*/*Rht-D1d*, are negatively linked to anther extrusion (Boeven et al., 2016b; Buerstmayr and Buerstmayr, 2016; Würschum et al., 2018) whereas this is not the case with *Rht24b* (Boeven et al., 2016b; Würschum et al., 2017a, 2018). One option for the parental lines is to select a male line with the wild type allele of *Rht1* and the height reducing allele *Rht24b*. The female line will combine the three reducing height alleles *Rht-B1b*, *Rht-D1d* and *Rht24b*, resulting in a shorter height compared to the male line (Würschum et al., 2018).

I.3.4. Methods of production of hybrid wheat

The production of hybrid seeds requires a directional cross between a seed parent (female parent) and a pollen donor (male parent). As wheat floral biology ensures complete self-pollination, the production of pollen by the female parent has to be prevented to avoid the female plants from pollinating themselves. Male sterile plant can be produced by different methods: emasculation, chemically induced male sterility, genetic, cytoplasmic and engineered male sterility (Singh et al., 2015). The system can only be commercially used if it prevents self-pollination of the female line, if the multiplication of the male sterile line is easily done at large scale and if the fertility restoration in the F_1 generation is total (Kempe et al., 2014).

I.3.4.1. *Hand emasculation*

The simplest way to obtain a male sterile plant is to emasculate the flower by removing manually the anthers a few days before anthesis. This method is

highly time consuming and cannot be used at a field scale in the case of wheat (Kempe and Gils, 2011; Singh et al., 2015).

I.3.4.2. *Self-incompatibility system*

Self-incompatibility (SI) is the inability of a plant to self-pollinate even if pollen and ovule are viable. The SI system is based on two lines which are homozygous for two different recessive S alleles. The F_1 hybrids are self-compatible and fertile. The major problem in this system is the maintenance of the parental lines (Kempe and Gils, 2011). However, whereas SI is widespread in grass and rye, it is absent in wheat. Introgression of genes controlling SI from wild relatives into the wheat genome is a possibility, but it does imply an increase in the selection cycle (Whitford et al., 2013).

I.3.4.3. *Chemical Hybridizing Agent*

Use of a chemical hybridizing agent (CHA) induces male sterility in wheat by causing physiological abnormalities and altering the pollen. CHA allows the production of a high number of parental combinations to evaluate the combining ability of the parents (Singh et al., 2010; Whitford et al., 2013). According to Virmani and Edwards (1983), an ideal CHA affects only the male organs and not the female organs, does not have any phytotoxic effects on the parents, is effective on a large panel of environments, has a flexible time of application, is environmentally safe, achieves the sterility in one treatment, does not affect the F_1 seed quality and the F_1 plant vigor. More than 40 chemicals have been patented such as auxin/anti-auxin, halogenated, aliphatic acids, gibberellins, etephon and arsenicals (Fu et al., 2014a; Singh et al., 2010). Most of these chemicals showed strong phytotoxic effects or worked on only a few of the genotypes (Whitford et al., 2013). More recently Monsanto developed Genesis® (clofencet) which was used in Europe and USA until 2007 (Cisar and Cooper, 2002). Currently Croisor®100 (sintofen), a plant growth regulator developed by Saaten Union Recherche, is the only gametocide agent used in Europe. Improvements still have to be made on decreasing the toxicity, the cost of the chemical, the ratio of male versus female lines and the

dependence of the environmental conditions (Fu et al., 2014a; Kempe and Gils, 2011). Moreover, male lines have to be removed after pollination to ensure the purity of the hybrid seed production. The male lines can be removed mechanically if they are taller than the female lines (Kempe and Gils, 2011).

I.3.4.4. *Cytoplasmic male sterility*

Cytoplasmic Male Sterility (CMS) is another possibility to produce hybrid wheat. A CMS system is a three lines system: a CMS line (A-line) which carries a sterile cytoplasm without a *Restorer of fertility* (*Rf*) gene, a male fertile maintainer line (B-line) carrying a normal cytoplasm and the same nucleus as the A-line and a restorer line (R-line) which carries a *Rf* gene and is used as the male parent to produce the fertile hybrid F_1 (Chen and Liu, 2014; Whitford et al., 2013) (**Figure I-2**).

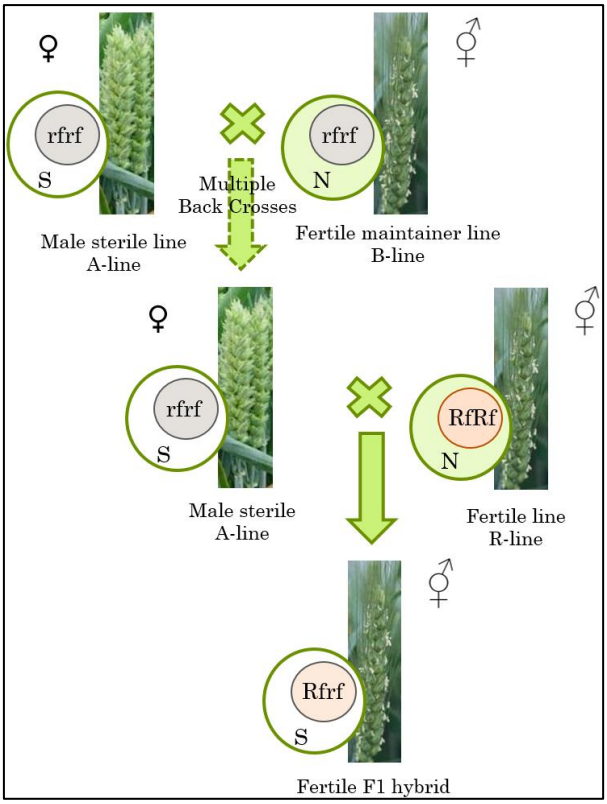


Figure I-2 Hybrid breeding system using cytoplasmic male sterility
The cell of each lines is represented by the cytoplasm (outer circle) and the nucleus (inner circle). “S” means sterile cytoplasm, “N” normal cytoplasm, “rf” non-restorer allele, “Rf” restorer allele

CMS has been described in over 150 different plant species (Wise and Pring, 2002). The first CMS system was discovered in onion by Jones and Emsweller in 1937. One of the most famous is the T (Texas)-cytoplasm for maize associated with the mitochondrial gene *T-urf13*. The high susceptibility to Corn Leaf Blight conferred by this T-cytoplasm has forced the breeders to use other type of sterile cytoplasms (Crow, 1998; Schnable and Wise, 1998). Today successful CMS systems are used to produce commercial hybrid seeds for rice, sorghum and sunflower (Perez-Prat and van Lookeren Campagne, 2002). However, few CMS hybrids have been registered due to the complexity of the system, the low yield advantages obtained and the high seed cost. In order to have a productive CMS system, breeders need to maintain the three different lines and the number of sources of restoration is limited, which can restrict the heterosis (Singh et al., 2015). Fertility restoration is not only under a complex genetic control, but also environmental control (Maan et al., 1984). Some restorers could be adequate for a certain kind of environment and some male sterile lines are easier to restore (Cisar and Cooper, 2002; Virmani and Edwards, 1983).

CMS can arise both spontaneously and following mutagenesis, or be the result of interspecific, intraspecific or intergeneric crosses. Numerous species related to common wheat (genera *Triticum*, *Aegilops*, *Hordeum* or *Secale*) have cytoplasm which could form the basis of the CMS. Singh, et al. (2010), counted more than 70 different male sterile cytoplasms but a lot of them are not usable because of biologically negative effects such as reduction of germination and seedling vigour. The first cytoplasm to be reported was that of *Aegilops caudata* by Kihara in 1951. Plants carrying *Ae. caudata* cytoplasm were partially female sterile and showed pistillody (Kihara, 1967a). The nucleus of *T. aestivum* has been introduced into *Ae. ovata* cytoplasm and a restorer was reported at the same time (Fukasawa, 1959). In 1962, Wilson and Ross reported the sterility conferred by *T. timopheevii* cytoplasm. In 1979, Mukai and Tsunewaki reported that cytoplasm from *Ae. kotschyi* and *Ae. variabilis* caused total sterility in wheat. Cytoplasm of 47 species belonging to the genus

Aegilops and *Triticum* have been classified into different plasmon groups according to their effect on the agronomic traits of common wheat (Tsunewaki, 2009; Tsunewaki et al., 1976, 1996, 2002). No cytoplasm has been found which gives an improvement in important wheat characters. *Ae. kotschyi* cytoplasm reduces dry matter weight and *Ae. variabilis* cytoplasm affects plant height, ear number and dry matter (Mukai and Tsunewaki, 1979). From a wider cross, the cytoplasm of *Hordeum chilense* causes sterility in common wheat and does not show important negative effect on wheat phenotype (Martin et al., 2008).

I.3.4.4.1. Mechanisms inducing male sterility

CMS is a maternally inherited condition caused by a defect in mitochondrial DNA resulting from DNA rearrangements. This leads to the expression of a protein causing male fertility (Hanson and Bentolila, 2004; Touzet and Meyer, 2014). CMS plants show a huge range of phenotypic abnormalities. In some cases, male sterility causes homeotic alteration: the stamen can be transformed into a petal (petaloid phenotype) or into a carpel (carpeloid phenotype). In other cases, CMS can just be expressed by the failure of the development of the pollen or the non-function of the pollen (Chase, 2007; Hanson and Bentolila, 2004). The CMS phenotype can be suppressed or counteracted by nuclear genes, known as *Rf* genes, specific for each CMS system (Chase and Gabay-Laughnan, 2004; Hanson and Bentolila, 2004).

Plant mitochondria are semi-autonomous organelles which have an essential role in ATP synthesis. Most CMS genes are chimeric resulting from structural mitochondrial genomic rearrangements that fuse together portions of two or more different genes to create a novel gene. Many CMS genes have been characterized as open reading frames (ORFs). The ORFs are the results of multiple recombination events which lead to the integration of some mitochondrial genes and genes of other origins into the mitochondrial genome. As a result of these rearrangements, ORFs may be fused to a promoter sequence or be located downstream of pre-existing active genes.

They will thus be expressed independently or co-transcribed with the upstream gene (Chase, 2007; Chase and Gabay-Laughnan, 2004; Chen and Liu, 2014; Hanson and Bentolila, 2004). Most of these genes are involved in ATP synthase or cytochrome oxidase (cox) subunits genes (Chen and Liu, 2014; Hanson and Bentolila, 2004). The ORFs encode for novel proteins which will interfere with mitochondrial function (Wise and Pring, 2002).

Four models have been proposed to explain the mechanism causing CMS.

- The first one is the cytotoxicity model in which the CMS protein kills the cells causing mitochondrial dysfunction in the sporophytic and gametophytic cells of the anthers (Chen and Liu, 2014).

- The second model is the energy deficiency model in which the cells fail to meet the energy requirements for male reproductive development. Many CMS proteins are mitochondrial transmembrane proteins. By integrating into the mitochondrial membrane, these proteins cause proton leakage and disruption of ATP production (Chen and Liu, 2014; Duroc et al., 2009; Rhoads et al., 1995). The “ATP hypothesis” has been questioned as respiratory mutants have been able to produce viable pollen despite a very low mitochondrial ATP production (Robison et al., 2009). This means the CMS must be causing a different disruption such as decrease in the production of a precursor for the outer layer of the pollen grain. But to date no such function has been discovered (Touzet and Meyer, 2014).

- The third model is the aberrant Programmed Cell Death (PCD) model. PCD is controlled by a mitochondrial driven signal and is essential in the plant developmental processes associated with senescence, seed germination or disease resistance (Chen and Liu, 2014; Ku et al., 2003). Production of viable pollen is also dependant on a well-controlled PCD. Degeneration of the cells of the tapetum is essential soon after pollen development is completed to allow the release of the pollen (Wu and Cheung, 2000). Premature or delayed PCD is a cause of male sterility (Chen and Liu, 2014; Ku et al., 2003; Wu and Cheung, 2000). The two principal signals are release of cytochrome c from the mitochondria to the cytosol and overproduction of the reactive oxygen species

(ROS) (Chase, 2007; Chen and Liu, 2014). ATP is needed to ensure PCD, and thus a decrease in the amount of ATP can cause an abnormal activation of the PCD pathway (Bras et al., 2005).

- The last model is the retrograde regulation model. Some mitochondrial genes are able to modulate the expression of certain nuclear genes (Ryan and Hoogenraad, 2007; Woodson and Chory, 2008). It has been shown that CMS genes can regulate the expression of nuclear MADS-box genes, which play a major role in flower development. In carrot carpeloid, the CMS phenotype is associated with the decreased expression of the two MADS-box genes, which control petal and anther formation (Linke et al., 2003). It seems that CMS is the result of a combination of the different models (Chen and Liu, 2014).

I.3.4.4.2. Mechanisms of cytoplasmic male sterility restoration

The expression of CMS gene can be suppressed or counteracted by the products of *Rf* genes. The restoration can involve one or several *Rf* genes. The first *Rf* gene cloned was *rf2*, able to restore maize CMS-T cytoplasm (Cui et al., 1996). *Rf2* code for an aldhehyde dehydrogenase (Liu et al., 2001). Eight other *Rf* genes have been isolated since *Rf2* in different species, most of them coding for pentatricopeptide-repeat (PPR) proteins (Chen and Liu, 2014). The PPR protein is a 35 amino acid motif repeated tandemly up to 30 times (Schmitz-Linneweber and Small, 2008). PPR motifs consist of two antiparallel α-helices which will bind the RNA. The result of this interaction is posttranscriptional mRNA processing such as editing, splicing, cleavage and degradation (Delannoy et al., 2007; Schmitz-Linneweber and Small, 2008). The exact function of the *Rf* genes is still unknown but their expression leads to the decrease of the expression of the CMS gene (Chase, 2007). It is thought that the PPR protein might bind to the transcript encoded by the CMS gene and hence might prevent its translation into protein (Chase, 2007; Schmitz-Linneweber and Small, 2008).

I.3.4.5. *Genic male sterility*

Genic male sterility (GMS), also called nuclear encoded sterility (NMS) has been found in many species such as tomato, barley, rice and wheat (Kempe and Gils, 2011). Sterility is caused by mutations in nuclear-encoded genes, the mutations being spontaneous or induced (ϒ irradiation for example) (Whitford et al., 2013). Male sterile mutant causes abnormalities of the development of sporophytic (affecting primarily the tapeta and the meiocytes) or gametophytic (affecting the development of microspores and pollen grain) anther tissues (Chen and Liu, 2014). In wheat, eleven genes inducing GMS have been identified. Three of them are dominant: *Ms2* and *Ms3* on 4DS and 5AS respectively (McIntosh et al., 1998) and *Ms4* on 4DS (Maan and Kianian, 2001). The other eight mutations are recessive. Seven of them are allelic at the *ms1* locus located on chromosome arm 4BS (Endo et al., 1991; Klindworth et al., 2002). The eighth gene *ms5* is located on 3AL (Klindworth et al., 2002). In a breeding process recessive genes, are desirable allowing an easier identification of the homozygous sterile progeny. The first monogenic recessive mutation leading to partial male sterility was identified in the UK cultivar Moulin (Trottet et al., 2010). However, the location has not yet been identified.

This system increases the choice of parental lines and avoids the negative cytoplasm effect when compared to CMS (Fu et al., 2014a; Whitford et al., 2013). The main problems are the maintenance of the male sterile parent, the multiplication and the selection of pure male sterile lines. In order to screen the sterile/fertile lines at a large scale, a male sterile locus tightly linked with a visual marker is desirable. For example *Ms2* is linked to the *Rht10* dwarfing locus (Whitford et al., 2013). As a result, male sterile line will be shorter than the male fertile line, which has to be removed. Production of a pure male sterile population can be facilitated by the use of the three lines XYZ system (Driscoll, 1972, 1985). This system is based on the addition of a wild relative chromosome to restore the fertility. The original system relies on the addition of chromosome 5R from *Secale cereale* L. to the male sterile Cornerstone

mutant (*ms1c*) (Driscoll, 1977). Chromosome 5R of rye, which restores fertility, was proposed for this system because it carries a dominant marker character, hairy peduncle, which facilitates seed separation. Nevertheless, selection can only be done at the adult stage. To allow earlier screening, a seed colour system has been developed. This system relies on the addition of chromosome 4E of *Agropyron elongatum* ssp. *Ruthenicum* Beldie (2n = 70) to the spontaneous Lanzhou mutant background (*ms1g*). Chromosome 4E carries a dominant male-fertility gene and a blue grain marker gene (Zhou et al., 2006). This gene has a "gene dosage effect" with deep, middle or light blue seeds related to the number of chromosome 4E present, which allows a quick seed selection. This system has been named 4E-ms. Other wild relatives are possible donors of a restorer chromosome such as *T. boeticum*, *T. monoccocum* and *Th. bessarabicum* (Buresová et al., 2015). The low inheritance of the blue seed character restricts the commercial development of this system, which is still an interesting option for recurrent selection and population improvement (Whitford et al., 2013).

I.3.4.6. *Environment male sterility*

In some cases, genic male fertility is controlled by photoperiod (PGMS) or by temperature (TGMS) or both of them (PTGMS). This system is composed of only two lines as no maintainer is needed, self-pollination of the male line occurring under specific environmental conditions. Hence, environement male sterility (EGMS) lines cannot be used broadly but are restricted to certain environments (Virmani and Edwards, 1983). A nuclear thermosensitive nuclear male sterile gene *wtms1* has been mapped on chromosome 2B of wheat (Xing et al., 2003). Male sterility is expressed when the temperature during spike differentiation is below 10°C. Three recessive PTGMS sensitive genes have been mapped and located on 5B (*wptms1*), 2B (*wtpms2*) (Guo et al., 2006) and 1BS (*wtpms3*) (Chen et al., 2011).

A two-line photoperiod-sensitive cytoplasmic male sterile (PCMS) system with the cytoplasm of *Ae. crassa* has been developed (Murai and Tsunewaki, 1993;

Murai et al., 2002). The alloplasmic line is sterile under long day conditions (more than 15 hours), showing homeotic transformation of the stamen into pistilloid structures (Murai et al., 2002). This system shows a high fertility restoration by the combined effect of short-day conditions and effective fertility-restoring gene(s). Restorer genes have been found on chromosome 7B of Chinese Spring (Murai et al., 2002) and on 4A, 1D, 3D and 5D of the cultivar Norin 61 (Murai, 1997).

I.3.4.7. *Engineered nuclear-encoded pollination control systems*

The biological techniques used to control pollination are complex and time consuming. Genetic modification system can be used to overcome these difficulties. Biotechnological approaches were described for the first time in wheat by De Block *et al.,* (1997) using the Seedlink® system of Bayer CropScience which is the only commercialized transgenic male sterility system (Kempe et al., 2014). The Seedlink® system relies on the expression of a cytotoxic bacterial ribonuclease from *Bacillus amyloliquefaciens* (barnase), coded by a *barnase* gene (Paddon and Hartley, 1985). This results in the destruction of the tapetum, which is essential in pollen development as a source of nutrients (Kempe and Gils, 2011). The maintenance of the male sterile line is done by a back cross with a non-transgenic plant leading to a population composed of 50% fertile plants. By coupling this system with a glufosinate resistance LibertyLink®, selection of male sterile plants is possible at a field scale (Whitford et al., 2013).

As the *barnase* gene is transmitted to the F_1 generation, fertility restoration in the hybrid is achieved by the expression of the *Barstar* gene carried by the father line. The *Barstar* protein inactivates the RNA formed by the activity of *barnase* by forming a one to one protein complex (Mariani et al., 1992). The *barnase-barstar* system needs two genetically modified parents implying breeding requires extra time (Kempe et al., 2014; Whitford et al., 2013). To overcome this barrier a recessive split gene system has been developed, in which the *barnase* gene is encoded by two non-overlapping sequences at

complementary loci (Gils et al., 2008). This system relies on the fact that co-expression of two *barnase* gene fragments in the same tissue is sufficient for RNAase activity. The system is still limited by the segregation of the *barnase* fragments in the gametes. To resolve this problem the two *barnase* segments have to be positioned in an allelic relationship on physically distinct homologous chromosomes ("isoloci") (Gils et al., 2008). Male sterile lines carrying the two "isoloci" are easily propagated by a recurrent parent carrying a single "isolocus". By linking the "isoloci" not present in the maintainer line, heterozygous male sterile lines can be easily selected (Kempe et al., 2014). However, this results in the elimination of half of the plants sown during application of the herbicide. During meiosis the two fragments of *barnase* segregate, the hybrid progeny inherits only of one segment and expresses normal fertility (Kempe et al., 2014).

I.3.4.8. *Transgenic system producing non-transgenic seeds*

A new approach using a transgenic system but producing non transgenic seeds has been developed successfully by DuPont Pioneer for the production of hybrid maize: the Seed Production Technology (SPT) (Pioneer Hi-Bred International, 2011). The SPT system uses a fertility restorer transgene in the maintainer line. This transgene is composed of three elements linked in a single construct and a single homozygous recessive locus for male sterility. The three elements include a single dominant wild type allele (MS) complementary to the recessive male sterility allele, a pollen germination inhibitor (PGI) transgene and a fluorescent seed colour marker gene (SC). The transgenic system (MS, PGI, SC) is restricted to the maintainer line. Only half of the SPT pollen grain will carry the SPT transgene (MS, PGI, SC) and the transgenic pollen is unable to germinate because of the presence of the PGI element. The pollen grains which are not carrying the STP transgene are able to fertilize the female line (which carries the *ms* gene). The presence of the seed colour marker (SC) in the transgene allows the detection of the seeds carrying the STP system which have to be separated from the male sterile lines. The female is non-transgenic and will carry a non-transgenic F_1 seeds after being pollinated by a

restorer line (Wu et al., 2016). This system has not been developed yet for hybrid wheat production. The first step is the identification of a suitable, non-conditional, nuclear-encoded recessive male sterile. A male fertility gene *MS1* complementing the functionally of *ms1d* has been identified (Tucker et al., 2017).

I.3.4.9. *Hybrid wheat production history*

Kihara (1951) was the first to report the existence of Cytoplasmic Male Sterility (CMS) in wheat by using the cytoplasm of *Ae. caudata*. In 1962, Wilson and Ross discovered that *T. timopheevii* induced male sterility in common wheat. These discoveries initiated CMS hybrid research in the USA and the first commercial variety appeared in 1975 on the American market. In 1973, the first Chemical Hybridization Agent (CHA) was developed in the USA. In 1982 Monsanto launched a hybrid wheat programme in the USA and Europe based on the CHA "Genesis®". But over time many breeding companies stopped their hybrid program because of the lack of knowledge and the seed production costs resulting from the complexity of the hybrid system (Singh et al., 2010). The production cost of one test hybrid has been estimated to be four times the cost of one yield plot (Longin et al., 2014a). Currently the majority of European hybrid wheat is produced by CHA with Croisor®100 (Longin et al., 2012). In India, hybrid wheat production is based on CMS, whereas in China the main method of production is photoperiodic sensitivity (Singh et al., 2010).Very few studies comparing the different systems exist (**Table I-2**).

Table I-2 Comparison of the different hybrid breeding system

Method	Advantages	Disadvantages
CHA	Very high potential for hybrid combination Easy to use Efficient seed production	Phytotoxicity Environmental toxicity Strong environment dependence of the male sterility Cost of the chemical
CMS	High potential for hybrid combination Natural male sterility Total sterility High hybrid purity	Three lines system implying long breeding cycle Need restorer allowing complete restoration Genotype and environment dependant Negative effect of the cytoplasm
GMS	High potential for hybrid combination Sterile and maintenance lines are the same	Remove 50% of the lines for seeds production Cost of seeds production
EMS	High potential for hybrid combination No need of maintenance lines	Instability of the male sterile phenotype Restriction to a given area
Transgenic	High potential for hybrid combination Total sterility High hybrid purity	Cost of the transgene development Acceptance by the society

In 2004, Adugna compared the efficiency of CMS (with *T. timopheevii* cytoplasm) and CHA (based on CH9832) systems for hybrid performance and hybrid seed production as well as performance of the hybrids. The CMS system appeared to be superior to the CHA for hybrid seed production because of the absence of chemical toxicity leading to a significantly higher outcrossing level, higher thousand seed weight (twice) and higher germination rate (twice) of the hybrid seeds (Adugna et al., 2004). Selection time is also an important criteria to take into account to evaluate the gain of each different system (Longin et al., 2012). The CMS system is one of the most time consuming as both parents have to be bred.

I.4. Utilisation of wild relatives to develop a hybrid wheat system

Wheat wild relative species are a huge reservoir of sterile cytoplasm, nuclear restorer genes, or genic male sterile genes (Sinha et al., 2013). Singh, et al. (2010), counted more than 70 different cytoplasms causing male sterility in common wheat. Each of these species are thus potential donors of the cytoplasm of the A-line. *Rf* genes are also present in the genomes of the wild species donor of the sterile cytoplasm as well as in the genomes of other wild relatives. In order to produce an interesting R-line, the *Rf* gene has to be introduced into the wheat genome. More broadly, the diversity of local cultivars and related species offers numerous sources of genetic variation to improve the narrow genetic base of cultivated wheat (Gill et al., 2011).

I.4.1. Gene pool concept and its consequence for crosses strategies

Gene pool classification is based on the genomic constitution of related species rather than formal taxonomy (Harlan and de Wet, 1971). The wheat gene pool is structured in three groups (**Figure I-3**). The primary gene pool (GP1) of *T. aestivum* is represented by species having homologous chromosomes to wheat: *T. urartu* (the donor of the A genome), *Ae. tauschii* (the donor of the D genome), cultivated *T. turgidum* (AABB), wild *T. dicoccoides* (AABB) and hexaploid landraces (AABBDD). Gene transfers from the species belonging to the GP1 to wheat are straightforward by using normal breeding methods, e.g. hybridization, leading to homologous recombination. The progenies are strong and fertile. The GP1 is the most used for crop improvement and many genes have already been transferred to commercial cultivars (Friebe *et al.,* 1996). The secondary gene pool (GP2) is constituted by species sharing at least one genome with *T. aestivum*. It includes the diploid species of the Sitopsis section of *Aegilops* and other polyploid *Triticum* species such as *T. timopheevii* or *T. zhukovskyi*. The offspring between common wheat and one species of the GP2 can be fertile despite the presence of reproductive barriers. Gene transfers from wild species of GP2 can be achieved by direct crossing if they are located on homologous chromosomes to wheat. They require cytogenetic manipulation if they are located on a non-homologous genome of wheat.

Lastly, the tertiary gene pool (GP3) is constituted by all other Triticeae species containing genomes other than A, B and D, such as *Secale* (RR) or *Thinopyrum* (EE). The offspring of these crosses are mostly sterile. In this case, gene transfers cannot be made by homologous recombination. Genetic transfers require special techniques to succeed such as ionizing radiation, tissue culture or bridging crosses with a member of the GP2 (Chaudhary et al., 2014a; Feuillet et al., 2008; Qi et al., 2007).

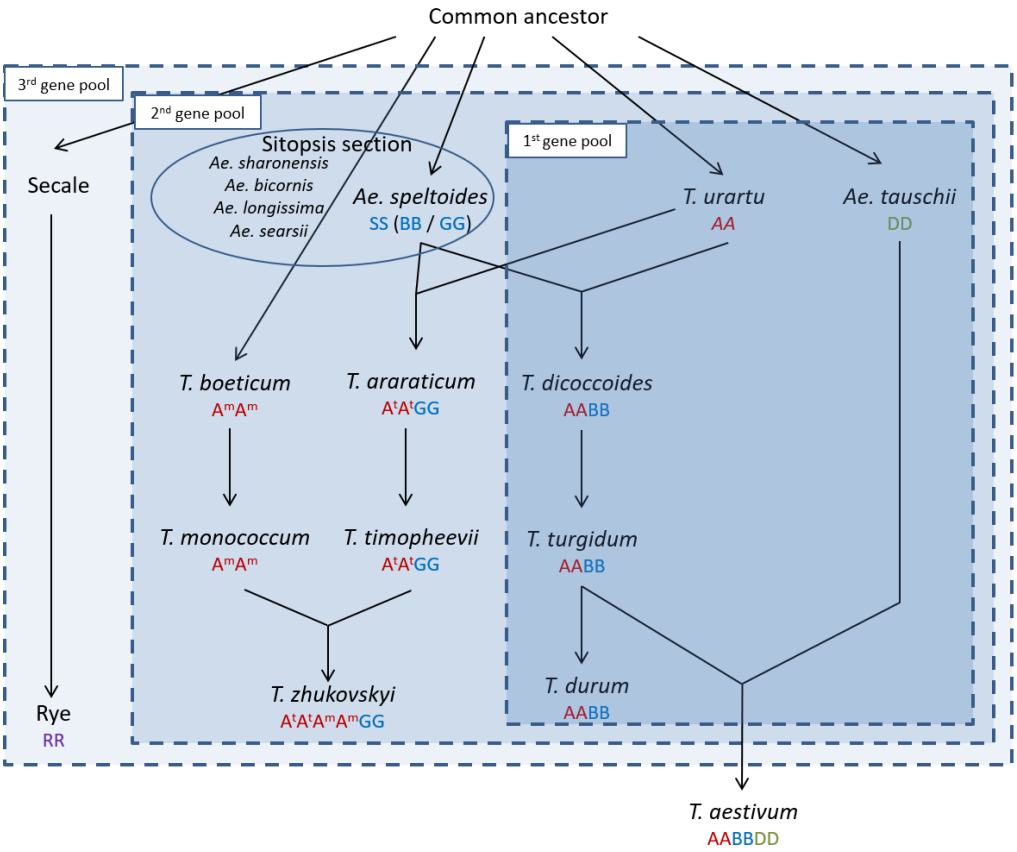


Figure I-3 Common wheat gene pools

Each gene pool is represented by square of different colour. The genomic composition is indicated below the name of the species

I.4.2. What can prevent the success of an interspecific cross

The major barriers to interspecific hybridization are classified into two groups: prezygotic (before fertilization) and postzygotic (after fertilization) barriers (Prohens et al., 2017).

I.4.2.1. *Prezygotic barriers*

Pre-zygotic barriers prevent fertilization and include differences in flowering time, failure of the pollen to reach or adhere to the stigma, improper germination of the pollen and abnormal growth of the pollen tube (Chaudhary et al., 2014a; Tonosaki et al., 2016). Heat treatment of the pistils or the pollen is one possible artificial technique to overcome this barrier in Liliaceae (Tonosaki et al., 2016).

It has been shown that the success of the cross between wheat and rye is genetically determined by two dominant loci *Kr1* and *Kr2* on the wheat genome (Lein, 1943). The genes *Kr1* and *Kr2* are located on chromosomes 5B and 5A, respectively (Riley and Chapman, 1967). The dominant alleles *Kr1* and *Kr2* reduce the crossability by inhibiting pollen germination and pollen tube growth (Lange and Wojciechowska, 1976). Most wheat cultivars carry the dominant alleles *Kr1* and *Kr2*, but Chinese Spring has the recessive alleles *kr1* and *kr2* showing higher crossability with rye (Riley and Chapman, 1967). Other crossability loci have been identified in the wheat genome: *Kr3* on 5D, *Kr4* on 1A and *Skr* on 5BS (Bertin et al., 2009). *Kr* genes do not affect only the crossability between wheat and rye, but also influence the crossability between wheat and others wild species such as *Hordeum vulgare* (Molnár-Láng, 2015).

I.4.2.2. *Postzygotic barriers*

Postzygotic barriers include gene-flow or incompatibilities acting after successful fertilization and formation of a zygote in the first or even the following generations.

I.4.2.2.1. Allopolyploidization: a genomic shock

The result of an interspecific cross is the cohabitation in the same nucleus of two or more different genomes, a stress period named genomic shock for the newly formed hybrid. Polyploidy is synonym of restructuration of the genome including changes in chromosomes number or structure (eg. translocation),

DNA sequence elimination and epigenetic alteration (altered methylation patterning or transposon activation) (De Storme and Mason, 2014; Feldman and Levy, 2005; Tonosaki et al., 2016).

The first consequence of the hybridization is the change of the architecture of the cell with an increase of its volume. This results in changes of the stoichiometry of the interaction between components of chromatin (Comai, 2005). Newly formed polyploid has to face difficulties at meiosis. Indeed, F_1 hybrids show partial to complete lack of pairing leading to the formation of unstable univalent instead of bivalent. Unbalanced chromosomes segregation (unidirectional segregation of the univalent) leads to the formation of aneuploid gametes. In extreme cases, the whole genome of one of the parents can be eliminated such as in the case of wheat x maize crosses, used for the production of doubled haploid wheat lines (Niroula and Bimb 2009). This chromosome elimination has been linked to the action of centromeric histone H3 (CenH3) in the interspecific cross *Hordeum vulgare* x *H. bulbosum* (Sanei et al., 2011). Loss of this protein causes inactivity of the centromere and so the failure to assemble kinetochores. Kinetochore is a protein complex assembled at each centromere to which microtubules attach during chromosomes separation. The results is the loss of parental chromosomes (De Storme and Mason, 2014). Genomic instability and meiotic disorders lead to hybrid lethality or sterility. Newly formed hybrids which went through this genomic conflict have to achieve harmonious function of the different genomes. Precise pairing of homologs is the key to achieve complete fertility and is achieved by DNA sequence elimination, chromosome rearrangements and gene silencing (Pikaard, 2001). Duplicated genes can either be eliminated or retained through sub-functionalization (joint functionality of a pair of duplicated genes) or neo-functionalization (acquisition of a novel function in one of the pair of duplicated genes) (Comai, 2005). DNA elimination occurred in a very short period of time after hybridization in wheat (Feldman et al., 1997; Ozkan et al., 2001; Salina et al., 2004), involving up to 14% of the genomic DNA (Shaked et al., 2001). Elimination of DNA sequence led to the increase of physical

divergence between the homoeologous chromosomes and then the stabilization of the allopolyploid by participating to the diploid-like meiotic behaviour (Feldman and Levy, 2005).

One main factor involved in changes in gene expression is epigenetics. In a synthetic hexaploid wheat *T. turgidum* x *Ae. tauschii* most of the genes are silencing caused by gene regulation and not by DNA loss (He et al., 2003). Aneuploidy can be a reason of epigenetic changes by exposing unpaired chromatins regions to epigenetic remodelling mechanisms (Comai, 2005). Epigenetic remodelling causes activation or suppression of genes expression. It has been reported that up to 13% of genomic loci can be affected by alteration in methylation (Shaked et al., 2001) and that about of 2% of the genes of newly formed tetraploid wheat had an altered expression (Kashkush et al., 2002). Moreover, it has been shown that epigenetic mechanisms are regulating the silencing of ribosomal RNA (rRNA) from one parental species (Preuss and Pikaard, 2007). Cytosine methylation caused rye rRNA silencing in presence of wheat genome (Houchins et al., 1997). Depending on the degree of homology of the different genomes, chromosome rearrangements can occur leading to the production of recombinant genomes.

All these changes inducing modification in gene expression and at the end phenotypic and morphological modifications. Inter genomic gene interactions lead to novel trait, as the unique properties of the gluten , product derived from the contribution of the three genomes of hexaploid wheat leading to this high baking qualities (Feldman and Levy, 2005).

I.4.2.2.2. Hybrid Incompatibility

One result of the genomic conflict into new polyploid is the hybrid incompatibility. This includes lethality and sterility.

Reproductive barrier in endosperm

It is known now that normal endosperm growth is playing a major role in the embryo development. The plant reproductive process leads to a double fertilization giving raise to the embryo and the endosperm. The endosperm results of the fertilization of the diploid polar nucleus of the central cell (2n) by the haploid sperm nucleus (n) (Kinoshita, 2007; Tonosaki et al., 2016). Endosperm is a triploid tissue which has functions of protection and nutrients storage for the embryo (Berger et al., 2008). Normal development of the endosperm requires two sets of maternal chromosomes and one set of paternal chromosomes. An unbalanced ratio (different from 2:1) leads to endosperm abortion (Johnston et al., 1980). Failure of endosperm development causes disruption of the nutrient supply to the embryo. In the case of crosses between parents of different ploidy levels the ratio does get unbalanced. One possible explanation is that the disturbance of the genome balance is caused by epigenetic mis-regulation on imprinted genes (Kinoshita, 2007). Genomic imprinting is an epigenetic mechanism that results in non-allelic gene expression depending on the parent origin. The parental genes differ to each other by DNA methylation or histone modification. It has been proposed, according to the "parental conflict" theory (Haig and Westoby, 1989), that the maternally expressed imprinted genes were limited the nutrient flow to the embryo whereas the paternally expressed imprinted genes were promoting the embryo growth (Köhler et al., 2010; Scott et al., 1998; Sekine et al., 2013).

To overcome this reproductive barrier, called triploid block (Köhler et al., 2010), the embryo can be rescued and put on a culture medium (Tonosaki et al., 2016). Rescues are performed by either directly transferring the excised embryo to an artificial medium or indirectly through flower (ovary) or immature seed (fertilized ovule) culture. The culture medium replaces the endosperm and provides nutrients to the developing embryo. Nevertheless it has been mentioned that triploid block was acting as a major reproductive barrier as the backcrossed seeds obtained from the F_1 hybrid and one of the

parent, will be mainly a nonviable progeny with an unbalanced chromosomes number leading to fertility issues (Köhler et al., 2010).

Hybrid necrosis

Once the endosperm barrier has been overcome, hybrid plants can face hybrid necrosis during its growth preventing its reproduction. Hybrid necrosis is characterized by slow growth, discoloration, dwarfism and lethality of the plant. Two dominant loci causing lethal necrosis have been found in the F_1 generation resulting of crosses between *Ae. bicornis* and *T. monococcum*, *Ae. umbellulata* and *T. monococcum* (Sears, 1944). In *Triticum* spp., lethal necrosis have been found controlled by two complementary dominant genes *Ne1* and *Ne2*, mapped on chromosomes 5BL and 2BS of common wheat (Chu et al., 2006; Tsunewaki, 1960). Hybrid necrosis is caused by an autoimmune-like response through epistatic interaction between two resistance (R) genes (Bomblies and Weigel, 2007). It has been proposed that *Ne2* was the same gene than *Lr13*, a resistance gene against leaf rust (Zhang et al., 2016). Hybrid necrosis can be easily overcome by the choice of parents which have a compatible combination of epistatic interaction (eg. *Ne1Ne1ne2ne2* or *ne1ne1Ne2Ne2*).

Sterility of the hybrid

Hybrids resulting from an interspecific cross are usually sterile. A model has been proposed to explain this phenomenon: the Bateson-Bodzhansky-Muller (BDM) model. The BDM model relies on interaction between at least two genes that have functionally diverged in the respective parental species (Johnson, 2010). A complete hybrid incompatibility is considered as the result of combined effect of numerous BDM interactions (Bomblies and Weigel, 2007). The sterility is caused by reduced chromosome paring during meiosis due to the different ploidy level or a low degree of synteny between the two parents.(De Storme and Mason, 2014). Hybrid sterility can be overcome by doubling the chromosome number using antimitotic agents such as colchicine (Dhooghe et al., 2011; Planchais et al., 2000). Embryo rescue might be a

necessary step as well. Hybrid sterility is well illustrated by the cytoplasmic male sterility (refer to Chapter I.3.4.4.1).

To circumvent pre-zygotic and post-zygotic barrier a bridging species can be used in the cross scheme. A first cross is done between this third species and one original parent it is compatible with. The second parent is crossed to the hybrid of the first cross (Prohens et al., 2017). Two successes of this strategy are: *T. dicoccoides* used as bridging species between *T. aestivum* and *Ae. umbellulata* (Sears, 1956b) and *T. turgidum* used as a bridging species between *T. aestivum* and *Ae. ventricosa* (Doussinault et al., 1983).

I.5. Wild genome introgression strategies

Gene transfer can be achieved by hybridization when the wild species is cross-compatible with wheat. The taxonomic relatedness, the level of polyploidy and the cross direction are essential elements in the success of introgression (Rieseberg and Wendel, 1993). There are different approaches for transferring desired traits from wild relative species to wheat, to produce intergeneric and interspecific hybrids.

I.5.1. Addition of the whole wild genome into the wheat genome

The first possibility is to transfer the whole genome of the wild species into common wheat. This approach allows the testing of the whole wild genome and is thus a preliminary step before starting an introgression program. F_1 hybrids between the wild species and common wheat are denominated as amphihaploids as they contain a haploid set of chromosomes of each of the parents (only homoeologues). For example, the F_1 resulting from the cross between common wheat (AABBDD) and rye (RR) will carry 28 chromosomes (ADBR). These amphihaploids can be fertile thanks to the close relationship between the chromosomes of these different species (Feuillet et al., 2008). Nevertheless, most of the time the F_1 hybrids are sterile because of the aberrant meiosis. Chromosome doubling by using colchicine is needed to transform the sterile amphihaploid into a fertile amphidiploid (Nemeth et al.,

2015). Spontaneous chromosome doubling can also occur as a result of unreduced gametogenesis (Pignone, 1993).

Transfer of the whole genome of rye into common wheat gave raise to Triticale (*X Triticosecale*). The first successful cross between rye and wheat led to sterile F_1 plants (Wilson, 1876). Chromosome doubling by colchicine treatment enabled the production of a fertile hybrid combining the qualities of the two parents, i.e. the high yield potential and good grain quality of wheat and the resistance/tolerance to biotic and abiotic stresses of rye (Lorenz and Pomeranz, 1974; Mergoum et al., 2009).

Another successful transfer of the whole genome is the production of synthetic hexaploid wheat resulting from the cross between tetraploid wheat (AABBB) and an *Ae. tauschii* (DD) accession. The hybrid produced is a perfect way to introduce new diversity into common wheat as all the chromosomes of the hybrid are homologous with the chromosomes of common wheat (Chaudhary et al., 2014a).

I.5.2. Production of wild addition/substitution lines

This approach consists of the production of addition or substitution lines by back crossing the amphidiploid produced with common wheat (Qi et al., 2007). A complete chromosome of the wild species can either be added to the wheat genome (addition line) or replace one wheat chromosome (substitution line). Production of addition lines can lead to aneuploidy (any change from the normal euploid state including addition and/or loss of one or more chromosomes of the wild type) causing sterility. Due to the presence of three genomes, common wheat tolerates the addition or deletion of a chromosome or part of a chromosome (Chaudhary et al., 2014a).

I.5.3. Production of translocation lines

The final step is the introgression of a fragment of interest of the wild species into the wheat genome. Ideally the wild species chromosome segment should

be as small as possible to avoid linkage drag, i.e., the introgression of undesirable or deleterious genes that are negatively influencing the phenotype. Linkage drag is responsible for limiting the use of alien genes in breeding programs (Zhang et al., 2017). In the Triticeae tribe and Poaceae family, gene synteny has been conserved throughout genome evolution. This conservation implies that a chromosome (or a part of a chromosome) of the basic genome is genetically related to a chromosome (or a part of a chromosome) in all other genomes of the Triticeae tribe (Ahn et al., 1993). If the gene of interest is located on a homologous chromosome to wheat, introgression can be done by homologous recombination. However, if the gene of interest is located on a homoeologous chromosome, recombination may have to be induced by chromosome engineering (Qi et al., 2007). Three methods can be used to induce homoeologous translocation: spontaneous translocation, radiation, and induced recombination (Zhang et al., 2017). Spontaneous transfer of a complete arm of a wild species can happen by exploiting the centric breakage-fusion behaviour of univalent (Sears, 1952). In a monosomic substitution line, the wild chromosome and the wheat chromosome are both present as univalent and can break at the centromere during meiosis. The broken arms can fuse leading to Robertsonian whole arm translocations (Qi et al., 2007). The best illustration of a spontaneous translocation is the wheat-rye T1BL.1BRS. Translocations can occur in terminal, intercalary, or centric positions. However, translocations in terminal or intercalary position are not common since homoeologous chromosomes between wheat and alien species in wide hybrids show a low pairing level. Another technique to induce homoeologous translocation is to expose the seeds to ionizing radiation (Sears, 1952). For example *Lr9*, a leaf rust resistance gene from *Ae. umbellulata*, was successfully transferred to common wheat (Sears, 1956b). Breakage of the alien and wheat chromosomes is completely random resulting in translocations that are usually genetically unbalanced and show low fertility. The last possibility to induce homoeologous recombination is by disabling the *Pairing homoeologous* (*Ph*) system (see paragraph below) which leads to a balanced genetic content.

An overlapping fragment strategy has been successfully developed in the past. This strategy is based on two different inter-crossing lines which carry the same target-gene. The two lines also have different and overlapping wild species chromosome fragments with the target gene in the middle. Offspring of the cross of these two lines could carry reduced wild species chromosomes fragments with the target gene and without the deleterious genes (Sears, 1981).

I.6. Pairing homoeologous locus

I.6.1. Diploid-like chromosome pairing during meiosis in wheat

Common wheat is an allohexaploid composed of three genomes (A, B and D), each containing seven homologous chromosome pairs. Thus the 21 pairs of chromosomes can be divided into seven groups of six chromosomes (one chromosome pair from each genome). Each chromosome pair is homoeologous to the two other pairs of chromosomes from the two other genomes. These homoeologous chromosomes are genetically related, i.e. they have a similar gene order but different repetitive content (Devos and Gale, 1993). Although each chromosome should theoretically be able to pair with one of the other four homoeologous chromosomes (equivalent chromosomes from the other two genomes), chromosome paring is in fact mostly restricted to homologues (for example chromosome 1A pairs with 1A and not with 1B or 1D). Wheat shows diploid-like chromosome pairing during meiosis with 21 bivalents observed at metaphase I (Riley and Chapman, 1958; Sears, 1976). The diploid-like behaviour of the polyploid is a requirement to avoid aneuploidy and homologous chromosomes mis-segregation leading to unviable gametes (Cifuentes et al., 2010).

Meiosis is a cell division process ensuring that the gametes carry the correct number of chromosomes and generates genetic diversity (Cifuentes et al., 2010). Meiosis is composed of two consecutive chromosome divisions: the reductional (segregation of the homologues) and the equational (segregation of the sister chromatids) division. Before meiosis DNA is replicated with the

formation of sister chromatids that remain linked together. In prophase I, homologs condense and become aligned. A proteinaceous structure known as the synaptonemal complex (SC) is assembled between the two homologs which become intimately aligned along their length leading to the formation of mature bivalents. When the SC is degraded, crossovers (COs) or chiasmata enable the homologues to remain associated at metaphase I and so segregate properly. Homologous chromosomes synapse and recombine at sites marked by the binding of the mismatch repair protein MLH1 (Double Hollyday Junction Marked to become COs) (Martín et al., 2014). COs result in reciprocal exchanges of DNA between homologous chromosomes. At least one crossover per chromosome is required and it must take place between a homologous chromosome pair. It is essential for the proper disjunction of the homologous chromosomes at the first meiotic division (Cifuentes et al., 2010). Prophase I ends with the breakdown of the nuclear envelope. The meiosis spindle is formed on which the kinetochore will be attached. In anaphase I homologs migrate toward opposite poles of the cell so that each gamete carries only a single copy of each chromosome. During the second meiotic division (mitosis-like division) the chromatids of each chromosome segregate so that each gamete carries only a single copy of each chromosome (Page and Hawley, 2003).

The diploid-like behaviour is predominantly controlled by the presence of the *Pairing homoeologous 1* (*Ph1*) dominant locus located on chromosome 5BL of common wheat (Okamoto, 1957; Riley and Chapman, 1958; Sears and Okamoto, 1958). The *Ph1* locus is defined as a deletion phenotype. A second locus (*Ph2*) was located on chromosome 3DS (Mello-Sampayo, 1971; Mello-Sampayo and Lorente, 1968; Upadhya and Swaminathan, 1963). *Ph2* is about half as effective as *Ph1* (Sears, 1976). *Ph1* and *Ph2* do not play the same role in synaptic behaviour. The *Ph1* locus is involved in the restriction of both synapsis and crossing over to homologous chromosomes, whereas the *Ph2* locus affects the progression and completion of synapsis (Martinez et al., 2001). Different mutants have been produced: *ph1b* (Sears, 1977) and *ph2a* by X-ray-induced

deletion (Sears, 1982), *ph2b* by chemical (Wall et al., 1971) in hexaploid wheat and *ph1c* by X-ray irradiation in tetraploid wheat (Giorgi, 1978). Other less-effective suppressors were discovered on several other chromosomes - 5AL, 5DL, 3AL, 3DL (Cuadrado et al., 1991). The *Ph1* locus has been the most studied up to now as it is considered as the only real "*Ph*" locus (Martinez et al., 2001).

I.6.1. The *Ph1* locus

It was suggested that the *Ph1* phenotype results from the activity of several genes as ethylmethane sulphonate (EMS) treatment failed to produce mutants with the complete *Ph1* deletion phenotype (Griffiths et al., 2006). Molecular analysis of the *Ph1* locus has defined it as a 2.5 Mb region containing a duplicated chromosome 3B segment carrying heterochromatin and *TaZIP4-B2* (originally termed Hyp3, UniProtKBQ2L3T5), inserted into a cluster of seven *cyclin dependent kinase 2* (*cdk2*)-like genes interspersed with methyl transferase genes (originally termed SpG, UniProtKB-Q2L3W3) (Al-Kaff et al., 2008; Griffiths et al., 2006; Martín et al., 2017). Only five cdk2-like genes are present on 5A and two on 5D which suggest that these clusters arose by different tandem duplication events (Al-Kaff et al., 2008). In the presence of *Ph1*, *cdk2*-like genes of 5A and 5D are not expressed, whereas some of them are transcribed when *Ph1* is absent, thus leading to the conclusion that *Ph1* is controlling all the *cdk2*-like genes (Al-Kaff et al., 2008). Genes within the wheat *cdk* locus show close homology to kinase Cdk2 from humans (Yousafzai et al., 2010), which is involved in the initiation of replication and is required in early meiosis (Al-Kaff et al., 2008). Cdk2 is involved in H1 phosphorylation and replication and chromatin condensation (Krasinska et al., 2008; Viera et al., 2009). For example increased *cdk* activity can be associated with premature chromosome condensation (Furuno et al., 1999). Another metaphase I expressed gene, *C-Ph1*, has been proposed has a candidate for *Ph1* within the 2.5 MB region (Bhullar et al., 2014) but this hypothesis has been rejected (Rey et al., 2017).

A recent study concluded that *Ph1* has a dual effect on synapsis and CO formation (Martín et al., 2017). It was shown that *Ph1* alters the levels of histone H1 CDK2-dependent phosphorylation, which affects pre-meiotic replication and chromatin structure (Greer et al., 2012). At the beginning of meiosis, chromosomes first decondense and then condense as the process of recognition and intimate pairing occurs between homologous chromosomes. The remodelling of chromatin on both homologues is coordinated so that they are in the same conformation at the beginning of pairing. In the absence of *Ph1*, chromatin remodelling can be initiated asynchronously and prematurely so that homologues are in different conformational states. Thus, a homologue would be just as likely to align intimately with a related chromosome as with its true homologue (Prieto et al., 2004). The timing of chromosome condensation during the onset of meiosis is thus an important factor in controlling chromosome pairing. In wheat, prior meiosis chromosomes associate intimately in pairs via their centromeres leading to the formation of the centromere bouquet at the early stage of the meiosis (Martinez-Perez et al., 2003; Prieto et al., 2004). Only homologous synapsis occurs at the telomere bouquet stage whereas homoeologous synapsis occurs later in meiosis, during the dispersion of the telomere bouquet (Martín et al., 2017). In the absence of *Ph1* homologous synapsis is delayed, giving an opportunity for homoeologous synapsis to occur after telomere bouquet dispersal (Martín et al., 2017). Thus *Ph1* is promoting more efficient homologous synapsis and not preventing homoeologous synapsis as was previously assumed. The second effect of *Ph1* is the prevention of MHL1 sites on synapsed homoeologues developping to become COs and thus preventing recombination (Martín et al., 2014, 2017). In the presence of *Ph1* mutant MHL1 sites do convert to COs and hence the number of COs will be lower than the number of MHL1 sites. By modulating the growth condition of plants (high nutrient composition and low temperature), the level of COs can be increased (Martín et al., 2017; Rey et al., 2018). ZIP4 is involved in the effect of *Ph1* on CO formation (Martín et al., 2017). However, the molecular mechanism of how the *Ph1* locus affects both synapsis and CO is not currently known.

One deletion mutant has been used worldwide in breeding programs to introgress diversity from wild relatives into wheat: Chinese Spring *ph1b* (Sears, 1977). Accumulation of intergenomic exchanges in this mutant through the generation has been observed, mostly between the A and D genome of wheat and aneuploidy can occur leading to a reduced fertility (Sanchez-Moran et al., 2001). A *Ph1* mutant exhibiting homoeologous COs in a wheat - wild relatives hybrid but showing reduced homoeologous synapsis and COs between the wheat genomes would be ideal. Development of chemical induced *Tazip4-B2* mutants has been studied to produce more stable mutant lines as compared to the whole *Ph1* locus being mutant. Indeed *Tazip4-B2* within the *Ph1* locus is involved in the suppression of homoeologous COs but not in homoeologous synapsis (Rey et al., 2017).

Chromosomes able to suppress the *Ph1* locus have been reported in different wild relative species: chromosome 5U of *Ae. umbellulata* (Riley et al., 1973), chromosomes 3E, 4E, and 5E of *Lophopyrum elongatum* (Dvořák, 1987) and chromosome $5M^g$ of *Ae. geniculata* (Koo et al., 2017). Two *Ph1* suppressors with major effect have been mapped on the *Ae. speltoides* genome: *Su1-Ph1* and *Su2-Ph1*, respectively on the 3S and the 7S chromosomes. A QTL with a minor effect was mapped on chromosome 5S and was designated *QPh.ucd-5S*. This QTL may primarily regulate recombination between homologous chromosomes and acts independently of the *Ph1* mechanism (Dvořák et al., 2006). *Su1-Ph1* has been introgressed into the hexaploid wheat Chinese Spring and into the tetraploid wheat Langdon, revealing that expression of *Su1-Ph1* requires expression of a complementary gene present in the *Ae. speltoides* genome (Li et al., 2017a). *Ae. mutica* is the second species to possess genes that completey supress the *Ph1* activity. The two genes have not been located yet but may correspond to *Su1-Ph1* and *Su2-Ph1* (Dover and Riley, 1972).

I.7. Gametocidal gene or chromosome

In most cases, during the backcross process to develop alloplasmic or wild relative substitution/addition lines, chromosomes of the wild species are lost.

However, certain chromosomes stay in the plant host. Such chromosomes or genes have been denominated “gametocidal” (Maan, 1975) or “cuckoo” (in reference to their preferentially transmission through the generations) (Miller et al., 1982) chromosomes or genes. Gametocidal (*Gc*) chromosomes were discovered in wheat in 1975 simultaneously by two different groups. Endo and Tsunewaki (1975) tried to introduce *Ae. triuncialis* cytoplasm into the common wheat genome, but after five generations of backcrosses the lines were still carrying a chromosome of *Ae. triuncialis*. By substituting a *T. aestivum* nucleus into *Ae. longissima* and *Ae. sharonensis* cytoplasm, only monosomic substitution or monosomic addition lines were produced despite multiple generations of backcrosses (Maan, 1975). When the hybrid between these backcrossed lines and common wheat was used as the female all the progeny were found to carry one or two *Aegilops* chromosomes. These lines were male fertile but showing an incomplete seed set after self-fertilisation. The selfed progeny were all disomic substitution or addition and were fertile (Maan, 1975). This results lead to the hypothesis that the gamete carrying the *Aegilops* chromosome has a gametocidal action on the gametes lacking this chromosome. Miller (1982) crossed common wheat and *Ae. sharonensis* to develop addition lines but all the backcrossed lines produced contained a chromosome of *Ae. sharonensis*, which was identified by cytological techniques to be the same chromosome in all the lines and defined as the *Gc* chromosome. Preferential transmission of one chromosome of the decaploid *Thinopyrum ponticum* has been reported as well (Scoles and Kibirge-Sebunya, 1983). Heterozygous plants for the *Gc* chromosome produce two types of gametes and only the one carrying the *Gc* chromosome are functional. Gametes containing the *Gc* chromosome are preferentially transmitted to the offspring by causing chromosome breakage in the gametes lacking this gene leading to chromatid fragments, dicentric chromosomes, chromosomes bridges, deletions and translocations (Finch et al., 1984a; King and Laurie, 1993; Nasuda et al., 1998). The gametocidal action on the female side results in limited seed set, while on the male side, it results in the occurrence of both normal and abortive pollen. It has been noticed that the chromosome

abnormalities are more important when the *Gc* chromosome is transmitted via the pollen (Endo, 1990).

A dual function model was proposed to explain the mechanism responsible for the preferential transmission of the *Gc* chromosome associated with two phenotypes (Endo, 1990). The first phenotype is denominated as “breaker” and induces chromosome breakage in gametophytes sporophytically. The second is the “inhibitor” phenotype which prevents chromosome breakage. The breakpoints of chromosome aberrations seem to be restricted to specific DNA sequences (Endo and Gill, 1996). A restriction-modification system has been proposed to explain this mechanism (Tsujimoto, 2005). The *Gc* gene produces two enzymes: a restriction enzyme (RE) and a modification enzyme (ME) such as a methylase. The RE cleaves specific restriction sites. Where *Gc* genes are homozygous, the restriction sites are protected by DNA methylation by the ME and hence the RE cannot cleave these sites. Where the *Gc* gene is hemizygous, the ME function is incomplete and chromosome breakage can occur. Gametes without the *Gc* genes are generated and the restriction sites are not protected. One hypothesis is that the RE is provided by other cells. Unmethylated DNA is broken and the gametes become abortive. The *Gc* factor acts in the first post-meiotic interphase before the S phase of the first pollen mitosis. The second pollen mitosis is arrested by chromosome breakage (Nasuda et al., 1998).

Currently, fourteen *Gc* chromosomes have been detected during the production of alloplasmic or addition lines in several *Aegilops* species (Endo, 1990, 2007): on chromosome 2S (Endo, 1990; Tsujimoto and Tsunewaki, 1984a, 1988) and 6S (Kota and Dvorak, 1988) of *Ae. speltoides*, on chromosomes $2S^{l}$ and $4S^{l}$ of *Ae. longissima* (Endo, 1985, 1990; Maan, 1975), on chromosome $2S^{sh}$ and $4S^{sh}$ of *Ae. sharonensis* (Endo, 1982, 1985; Finch et al., 1984b; Miller et al., 1982), on chromosome $2C^{c}$ of *Ae. cylindrica* (Endo, 1979, 1988), on chromosome 3C of *Ae. caudata* (Endo and Katayama, 1978), on chromosome $3C^{t}$ of *Ae. triuncalis* (Endo, 1978, 1982; Tsujimoto and Noda,

1988) and on chromosome 4M^{g} of *Ae. geniculata* (Kynast et al., 2000) (**Table I-3**).

Table I-3 Gametocidal chromosomes identified in *Aegilops* species with their symbol according to (McIntosh et al., 1998)

***Aegilops* species**	***Gc* chromosome**	***Gc* symbol**
Ae. caudata	3C	
Ae. cylindrica	2C	
Ae. geniculata	4M^{g}	
Ae. longissima	2S^{l}	*Gc2-S^{l}1a*
Ae. longissima	4S^{l}	
Ae. sharonensis	2S^{h}	*Gc1-S^{l}1*
Ae. sharonensis	4S^{h}	*Gc2-S^{l}1b* or *Gc2*
Ae. speltoides subsp. *aucheri*	2S	*Gc1a*
Ae. speltoides subsp. *ligustica*	2S	*Gc1b*
Ae. speltoides	6S	
Ae. triuncialis	3C	*Gc3-C1*

In the case of chromosome 6S of *Ae. speltoides* where the chromosome breakage takes place in the gametes carrying the *Gc* chromosome, this chromosome won't be included in the following model (Kota and Dvorak, 1988). The *Gc* chromosomes belong to three different homoeologous groups (2, 3 and 4) and each group has a different mode of action (Endo, 1990). The mode of action of *Gc* chromosomes was studied by using double monosomic addition (or substitution) lines of different *Gc* chromosomes (Endo, 1982, 1985). A double monosomic addition of two *Gc* chromosomes with different modes of action does not show a better fertility than the respective monosomic addition. On the contrary, a double monosomic addition of two *Gc* chromosomes with the same mode of action does show better fertility than the respective monosomic addition because of the pairing between the two *Gc* chromosomes (Endo, 1990). The *Gc* chromosomes in the same homoeologous group have the same mode of action: the first group is

composed of the *Gc* chromosomes of *Ae. triuncialis* ($3C^t$), the second group by the *Gc* chromosomes of *Ae. longissima* ($2S^l$), *Ae. speltoides* (2S) and A*e. sharonensis* ($2S^{sh}$) and the third group by the *Gc* chromosomes of *Ae. longissima* ($4S^l$), *Ae. speltoides* (4S) and A*e. sharonensis* ($4S^{sh}$) (Endo, 1990; Niranjana, 2017). It seems that the *Gc* genes of group 4 are epistatic to the genes of the group 2. The *Gc* chromosomes of group 4 seem to have two separate *Gc* genes, one of which is the same as that on the 2S chromosome. The *Gc* gene of *Ae. triuncialis* has a totally different mode of action and does not interact with the *Gc* chromosomes of the two other groups (Endo, 1982, 1985). While the *Gc* chromosomes of *Ae. triuncialis and Ae. cylindrica* only cause breakage in the gametes, the *Gc* chromosomes of *Ae. sharonensis*, *Ae. speltoides* and *Ae. longissima* can also induce breakage in the zygotic cells (King and Laurie 1993, Endo 1988b). The relationship between the *Gc* chromosome of *Ae. cylindrica* and *Ae. geniculata* have not been studied (Tsujimoto, 2005).

The *Gc* chromosomes of *Ae. sharonensis*, *Ae. longissima* and *Ae. speltoides* have an intense gametocidal action, particularly *Gc* chromosomes 2S of *Ae. speltoides* and $4S^{sh}$ of *Ae. sharonensis* (Endo, 1982, 1985; Maan, 1975; Tsujimoto and Tsunewaki, 1984a). Gametes without the *Gc* chromosome will suffer severe chromosome breakage and become sterile. However, *Gc* chromosome of *Ae. cylindrica* and *Ae. triuncialis* have a milder gametocidal action (Endo, 1988). Gametes lacking the *Gc* chromosome suffer slight chromosome damage, can be fertilized and the *Gc* chromosomes are not selectively retained. Thus, complete sets of addition lines have been developed for *Ae. longissima* (Feldman, 1975; Friebe et al., 1993) and *Ae. speltoides* (Friebe et al., 2000) but not previously for *Ae. sharonensis*. Not all genotypes of the same wild species carry the Gc chromosome (Miller 1983) and allelic variation within a species has been noticed (Endo 1985). In addition, where the *Gc* chromosome is heterozygous, a proportion of non-carrier gametes can survive (Marais 2010). Moreover, the severity of the gametocidal action is also dependent on the wheat background. For example, the *Gc* chromosome of *Ae. cylindrica* has a complete gametocidal action and is preferentially transmitted

to the next generation in a Jones Fife background, whereas no preferential transmission was observed in a CS background (Endo, 1988). A suppressor gene, *Igc1*, was reported in the cultivar Norin 26 against the 3C *Gc* chromosome of *Ae. triuncialis* (Tsujimoto and Tsunewaki, 1985a). In the presence of *Igc1*, the Gc chromosome 3C is not preferentially transmitted but still induces chromosome mutations (Nasuda et al., 1998). *Igc1* is a dominant gene located in the pericentromeric region of chromosome 3B (Yamano et al., 2010). *Igc1* does not suppress the action of *Gc* genes from *Ae. sharonensis*, *Ae. longissima* and *Ae. speltoides*.

From a breeding point of view *Gc* chromosomes can be a barrier and an advantage. *Gc* chromosome can be an obstacle to introgress a fragment of wild relative into wheat. For example, rust resistance genes have been transferred from *Ae. speltoides* to common wheat, but these introgressions were not usable by the breeders because of the association with the Gc gene affecting plant development, fertility and seed quality (Marais and Pretorius, 1996). In order to remove the *Gc* gene, homoeologous pairing was induced to create recombinants lacking the *Gc* genes but carrying the resistance genes (Marais et al., 2010). On the other hand, *Gc* chromosomes induce chromosomal rearrangements generating new variation in the germplasm (Endo, 1990, 2007). *Gc* chromosome 3C of *Ae. triuncialis* is able to induce chromosome breakage in chromosome 1R of rye (Endo et al., 1994). Using this property, resistance genes for wheat rust were transferred from chromosome 1R into wheat (Masoudi-Nejad et al., 2002). The *Gc* chromosome of *Ae. geniculata* (syn. *Ae. ovata*) was used to induce chromosomal rearrangements in Triticale (Kwiatek et al., 2016). The *Gc* chromosomes can be used as well as a tool to enhance the transmission of an interesting traits through the generations. For example, a 4D.4S^{sh} wheat/*Ae. sharonensis* translocation line with the dwarfing gene *Rht2* on chromosome 4D was produced. This line ensures the constant transmission of the *Rht2* gene (King et al., 1996). Another utility of *Gc* chromosomes is the production of deletion stocks for physical mapping purposes (Endo, 1990). More than 400 deletions lines of Chinese Spring have

been produced by using the *Gc* chromosome of *Ae. cylindrica* (Endo and Gill, 1996). Deletion mapping unlike genetic mapping does not require allelic variation and thus can be carried out on a few individuals.

I.8. Characterization of introgression of wild relatives into wheat

I.8.1. Cytogenetic techniques

Cytogenetic techniques are one tool to efficiently detect and characterize the products of hybridization between wheat and wild relatives. During the late 1960s and early 1970s, chromosome banding techniques were developed by using DNA-binding chemicals to obtain reproducible banding pattern unique for each chromosome. Each band has a characterized location, size and staining intensity. The banding allows the identification of each chromosome, the accurate pairing (as the banding pattern is the same for homologous chromosome) and the detection of chromosomal aberration. The various chromosome banding techniques include Q-bands, G-bands, C-bands, R-bands, N-bands, H-bands, and T-bands (Kannan and Zilfalil, 2009). The Giemsa C-banding technique, which stained constitutive heterochromatin, was developed to identify and characterize individual wheat chromosomes of Chinese Spring (Gill and Kimber, 1974). Wheat/rye introgressions were successfully characterized using the diagnostic terminal bands of rye chromosomes (Friebe and Larter, 1988; Lukaszewski and Gustafson, 1983; Seal, 1982). However, chromosome banding reaches its limit when the chromosomal segment of interest lacks diagnostic bands.

In order to directly visualize introgressions of the wild relatives into wheat genome *in situ* hybridization (ISH) techniques were developed (Rieseberg et al., 2000). In this technique genome-specific DNA probes are used to paint the karyotype of the hybrid at the metaphase stage (Chaudhary et al., 2014b). The first probes were radioactively-labelled probes (Buongiorno-Nardelli and Amaldi, 1970). Later, probes with a marker molecule (fluorophore) were developed. The fluorescent signals are visualized by using a fluorescent microscope. Genomic *in situ* hybridization (GISH) uses total genomic DNA as a

probe. If the two parental species are distant enough, the repeated sequence of the two genomes are different enough to be distinguished. The DNA from one parent is labelled as probe while the total DNA of the other parent is used as a block. GISH allows the determination of the genetic constitution of a hybrid and the detection of wild relative introgressions into wheat. GISH was first applied on a *Hordeum chilense x S. africanum* hybrid (Schwarzacher et al., 1989). Wheat/rye translocations have been detected using a biotin-labelled DNA probe hybridizing to the entire length of all rye chromosomes (Lapitan et al., 1986). Multicolour GISH (mcGISH) uses a different probe for each genome present in the hybrids leading to a more precise analysis of the genome composition. This has been successfully used in the localization of *Am. muticum* (King et al., 2017) and *Ae. speltoides* (King et al., 2018) fragment into wheat. In order to precisely identify each chromosomes fluorescent *in situ* hybridization (FISH) was developed. FISH techniques increase the resolution of characterization by allowing detection and characterization of the wild genome across the whole wheat genome on the basis of divergent and dispersed repetitive sequences. The main barrier is the development of suitable specific probes between the two species studied. FISH was first successfully developed to identify rye chromosomes in wheat-rye hybrids using the wheat ribosomal RNA genes and 120 bp repeated DNA family of rye labelled with biotin-6-dUTP (Yamamoto and Mukai, 1989). Several probes have been developed such the psA1 repeat (Rayburn and Gill, 1985) used to identify the D genome chromosomes, the psc119.2 rye subtelomeric repeat (Bedbrook et al., 1980) to identify the B genome chromosomes and pTa71 DNA clone (Gerlach and Bedbrook, 1979) to identify nucleolar organizing regions on satellite chromosomes. These probes also have been used to characterize different wheat wild relative introgression lines. By combining the GISH and FISH methods, wild relative/wheat translocations have been located and identified for *Th. bessarabicum* (Grewal et al., 2017; Patokar et al., 2016), *Agropyrum cristatum* (Liu et al., 2010) and rye (Szakács et al., 2004).

Cytogenetic methods are essential to check the genomic constitution of hybrids but they are highly time consuming which limits their use on a high throughput scale. Moreover, some introgressions can be missed because of their very small size (Lukaszewski et al., 2005; Rey et al., 2015). High throughput and high resolution detection can be achieved by the development of molecular makers.

I.8.2. Molecular markers

Molecular markers have been used for many reasons in plants such has genetic and association mapping with QTL identification, genomic based breeding with marker assisted selection or genetic diversity analysis (Hayward et al., 2015). A wide range of molecular markers have been developed during the last four decades. The first generation of markers was the Restriction Fragment Length Polymorphisms (RFLP) (Botstein et al., 1980) which provided an ability to detect the occurrence of DNA fragments of different lengths after restriction digestion of genomic DNA from various sources. RFLPs are high reproducible, codominant, highly locus specific, very informative and do not need prior sequence information. However, RFLPs are low throughput markers (Grover and Sharma, 2016). RFLPs have been used to characterize and map a cryptic introgression from *Ae. geniculata* with rust resistance gens into common wheat (Kuraparthy et al., 2007).

The second generation of markers are PCR-based markers and include Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990) and Amplified Fragment Length Polymorphism (AFLP) (Vos et al., 1995). RAPDs are DNA fragments produced by PCR amplification using a single primer of arbitrary nucleotide sequence. RAPDs have been widely used for species with little or no genomic resources available. RAPD markers are dominant so the homozygous individuals cannot be distinguish to the heterozygous, they are not reproducible and not very reliable (Ellsworth et al., 1993). A fragment of chromosome 1RS of rye was identified in wheat by using RAPD markers (Iqbal and Rayburn, 1995). AFLPs markers are based on the selective PCR

amplification of restriction fragments from a complete digest of genomic DNA. AFLP are multi-locus markers, highly reproducible, very reliable and cost effective. AFLP markers were linked with the *Lr19* resistance gene transferred from *Thinopyrum* into wheat (Prins et al., 2001). While RAPD and AFLP markers are PCR-based markers for random genome profiling, microsatellite (SSR) markers target specific genome sites. SSRs (Tautz, 1989) represent a class of repetitive sequences widely distributed in all genomes. SSRs contain repeats of a sequence motif 1-6 bp in length. SSRs are highly polymorphic, locus-specific and co-dominant (Mason, 2015). SSR markers have thus been extensively used in plant breeding. SSRs markers were used to identify the *Pm12* resistance gene transferred from chromosome 6S of *Ae. speltoides* to chromosome 6B of common wheat (Song et al., 2007) and have also been used to construct a consensus map for common wheat (Somers et al., 2004).

The last generation of markers involves sequence-targeted techniques and allows high throughput analysis. It includes Single Nucleotide Polymorphisms (SNPs) (Rafalski, 2002), Diversity Array Technology (DArT) (Jaccoud et al., 2001), Kompetitive allele specific PCR (KASP) markers (He et al., 2014) and Genotyping by Sequencing (GBS) (Elshire et al., 2011). SNP markers are the most abundant form of genetic variation in nature. Their occurrence in coding genomic regions can be associated with phenotypic changes. They are mostly bi-allelic and are the result of insertion, deletion or substitution of nucleotides. SNPs are highly reliable, abundant, uniformly distributed throughout the genome, reproducible and transferrable (Rafalski, 2002). Next Generation Sequencing technologies (NGS) have revolutionized genomics approaches and dramatically reduced the sequencing cost, leading to the high throughput discovery of SNPs (Grover and Sharma, 2016). Several platform have been developed for wheat allowing for genotyping of thousands of SNPs simultaneously such as the 9K (9000 SNPs) and 90K iSelect Illumina (Cavanagh et al., 2013; Wang et al., 2014a) or the Axiom 820K and 35K (Allen et al., 2013; Winfield et al., 2012). SNPs have mostly been developed for the wheat genome and few of them have been designed for the characterization of wild relative

introgression (Wulff and Moscou, 2014). However, a high density Axiom SNP genotyping array for hexaploid wheat and its secondary and tertiary gene pool have been developed (Winfield et al., 2016).

The KASP assay utilizes a novel homogeneous fluorescent genotyping system (He et al., 2014). KASP markers are suitable for selecting for a single gene or trait from a large number of genotypes making SNPs very useful in gene mapping. KASP assays are fast to run and easy to interpret, making the development of genotyping assays based on KASP technology desirable. Successful KASP SNP markers have been developed to map two rust resistance genes from chromosome $5M^g$ of *Ae. geniculata* introgressed onto chromosome 5B of common wheat (Tiwari et al., 2014). KASP markers closely linked to *Pm53*, a powdery mildew resistance gene, from *Ae. speltoides*, have been developed improving the introgression process of this gene in common wheat (Petersen et al., 2015).

GBS is an approach identifying and genotyping thousands of SNPs simultaneously. It reduces the complexity of genomes followed by targeted sequencing of reduced proportions. GBS is a very low cost method which requires less DNA and does not require prior knowledge of SNPs, avoiding ascertainment bias of SNPs in new germplasm. Initially the GBS approach used a single restriction enzyme to capture the genomic sequence between restriction sites (Elshire et al., 2011). The choice of the restriction enzyme is very important as is it used for masking the repetitive regions. A two restriction enzymes approach has now been developed, generating suitable and uniform complexity reduction of complex genomes (Poland et al., 2012). GBS and array derived SNP markers from the Illumina iSelect 90K were developed to map the stem rust resistance gene *Sr42* on chromosome 6D of wheat. Some of the SNPs were then converted into KASPs to be used for marker assisted selection (Gao et al., 2015).

DArT is a hybridization based method involving genotyping of several hundred polymorphic loci spread over the genome without prior sequence information in a single assay. DArT markers are bi-allelic, scoring the presence or absence of a single base pair. They are very reliable, cost effective and very useful for genetic diversity analysis (Jaccoud et al., 2001). DArT markers have been used successfully in wheat for genotyping, genetic mapping and genetic diversity assessment (Akbari et al., 2006; Cabral et al., 2014; Iehisa et al., 2014; Orabi et al., 2014) and also in wild relatives (Alheit et al., 2014; Bolibok-Brągoszewska et al., 2014; Peleg et al., 2008).

I.9. Hypothesis and objectives of the thesis

Among the large panel of methods for hybrid wheat seed production, cytoplasmic male sterility is one of the most widely discussed technologies (Whitford et al., 2013). Many cytoplasm causing male sterility from the wild relatives have been introduced successfully into common wheat, but the *T. timopheevii* plasmon has been widely spread used because of the unfavourable effects of other cytoplasms (Koekemoer et al., 2001; Virmani and Edwards, 1983). Initial study done on the restorer lines revealed that restorer factors were present in various cereals species but that the restoration was often incomplete and relied on environmental and epistatic factors (Curtis and Lukaszewski, 1993; Johnson et al., 1967; Ma et al., 1995; Maan et al., 1984). Incomplete fertility restoration counteracts the effect of heterosis on grain yield, which is a major motivation for hybrid wheat breeding.

The research presented in this PhD thesis aimed to study the wheat wild relatives as source of improvement of the CMS system. The cytoplasm of two species, i.e. *T. timopheevii* and *Ae. sharonensis*, were studied (**Table I-4**). The PhD thesis was divided into seven different projects. The progress of the PhD was strongly dependant on the availability of the material tested.

Table I-4 Organisation and time scale of the PhD thesis

Cytoplasm	Chapter	2015	2016	2017	2018
T. timopheevii	**3**- Test of wheat/wild relatives amphidiploids				
	4- Study of *S. anatolicum* genome				
	5- Development of *Secale* introgression lines				
	6- Test of wheat/rye introgression lines				
	7- Test of wheat/*T. timopheevii* introgression lines				
	8- Test of wheat/*Ae. sharonensis* amphidiploid				
Ae. sharonensis	**9**- CMS system based on *Ae. sharonensis* cytoplasm				

The first part of the thesis (**Chapters 3** to **8**) was focused on the cytoplasm of *T. timopheevii* and the discovery of a total, monogenic and stable restoration. Two mains hypothesis were established:

- Unknown restorer genes causing complete and stable restoration might be present in the wheat wild relatives
- The reduction of the segment of the wild genome carrying the restorer gene introgressed into wheat might cause an increase in the level of restoration of fertility

As a first step we screened thirteen different species for their ability to restore the cytoplasm of *T. timopheevii* (**Chapter 3**). For that purpose, we used as pollinator a subset of the wheat / wild relatives available at the Nottingham/BBSRC Wheat Research Centre (University of Nottingham). F_1s

having wheat/*S. anatolicum* amphidiploid were fertile. We carried to carry on the progenies of the fertile F_1s to study in more details the ability of *S. anatolicum* genome to restore *T. timopheevii* (**Chapter 4**). In parallel, an introgression program of the genome of *S. anatolicum*, *S. iranicum* and *S. vavilovii* into wheat genome was deployed using the *Ph1* mutant lines (**Chapter 5**). In 2017, the wheat/rye and wheat/*T. timopheevii* introgression lines produced by the Wheat Research Centre became available and were thus tested on CMS lines (**Chapter 6** and **Chapter 7** respectively). As the wheat/rye and wheat/*T. timopheevii* introgression lines were available late in the project, we decided to test the genome of another species. The presence of restorer factors into *Ae. sharonensis* genome was studied in **Chapter 8**. The use of a gametocidal mutant line was needed to remove the *Ae. sharonensis* gametocidal chromosome and isolate the restorer chromosomes.

The second part of the project (**Chapter 9**) consisted of a study of a CMS system based on the cytoplasm of *Ae. sharonensis*. This new CMS system could be a potential alternative to *T. timopheevii* cytoplasm in case no monogenic restorer factor causing total restoration was found. Two breeding strategies were carried out at the same time to produce on one hand the wheat alloplasmic line with the cytoplasm of *Ae. sharonensis* considered as the A-line and on the other hand the restorer line.

II.

II. Chapter 2: General Material and Methods

II.1. Plant material

II.1.1. Seed germination

Seeds were sterilised with 1 mL 5% ClNaO (10% solution-sigma Aldrich, Appendix 1), followed by shaking for 15 minutes. The seeds were rinsed twice with fresh sterile water and then transferred into a Petri dish on filter paper dampened with fresh sterile water and kept in a dark room for 3 days at 4°C. If the seed did not germinate, it was sterilized one more time and the embryo was rescued and placed on a culture medium (MS + 2% sucrose + 1% agarose, Appendix 1) under sterile conditions. Once the seed germinated, it was sown into soil (John Innes compost No. 2).

II.1.2. Vernalisation

Three or four days after sowing, the plants were transferred to vernalisation (8°C, 12 hours of light per day). Spring wheat plants were vernalized for four weeks, winter wheat and wild accessions for eight weeks, amphidiploids for six weeks and backcrosses from four to eight weeks depending on the backcrossing generation.

II.1.3. Glasshouse conditions

At the end of the vernalisation, plants were potted out in individual 2 L pots in the glasshouse (16 hours light per day at 21˚C).

II.1.4. Crossings

Emasculation of the head of the female parent was done one to three days before anthesis, when the spike started to emerge from the flag leaf, while the anthers were small and green and the stigma was tight. The florets in the centre of each spikelet were removed with a pair of forceps and only the primary and secondary floret were retained for emasculation. The three anthers of each floret were removed using a pair of forceps. The emasculated

spike was covered by a crossing bag to avoid cross-pollination. Two or three days after emasculation when the stigma became fluffy and was judged ready for pollination, yellow and round shaped anthers from the male parent were used to pollinate the receptive stigma. The spike was bagged again.

II.2. DNA extraction

Six pieces of young leaf (3 cm length) were collected into a 1.5 mL tube and placed in liquid nitrogen. A stainless steel ball (5 mm) was added to the tube and the samples ground in a Qiagen Retsch mill (Type MM 300) at a frequency of 25 Hz/second for 6 minutes. 80 µL of extraction buffer (Appendix 1) was added to the tubes, which were shaken thoroughly before being incubated at 65°C for 1 hour. Tubes were then put on ice for 15 min to allow precipitation before adding 300 µL of 6M ammonium acetate (Appendix 1). The samples were then spun down and the supernatant was transferred to a new 1.5 mL tube and 600 µL of iso-propanol was added. The samples were mixed thoroughly and placed at 4^{o}C for 1 hour to allow precipitation. The tubes were then centrifuged for 10 minutes at 13,000 rpm and the supernatant was removed. The pellet was washed with 300 µL of 70% ethanol. The tubes were centrifuged for 3 minutes at 13,000 rpm and the supernatant was tipped off. The DNA pellet was dried at 37°C for about 5 minutes. The pellet was then suspended in 100 µL of fresh water. The tubes were stored at -20°C. The DNA quantity was determined using a Qubit 2.0 fluorometer 5. The working solution was made in the ratio of 0.5 µL reagent dye: 200 µL buffer. Thin-wall PCR tubes (Invitrogen cat#Q32856 x500 tubes) were used. For each sample, 199 µl of working solution was added to 1 µl of sample. The Qubit was set up to carry out the DNA quantification. Once the DNA concentrations were determined, the remaining DNA was stored in the -20°C freezer.

II.3. Genomic *In Situ* Hybridization (GISH) and Fluorescent *In Situ* Hybridization (FISH)

II.3.1. Preparation of the probe

II.3.1.1. *DNA purification*

DNA, extracted according to the protocol explained in the section II.2, required an extra step of purification. After the pellets were re-suspended in 100 – 200 µL of de-ionised H_2O 200 µL of phenol chloroform was added to each tube. The tubes were mixed to obtain a cloudy solution and then were centrifuged for 5 minutes at 13,000 rpm. The supernatant was transferred in a new tube to which 20 µL of 3M NaAC (pH 5.2) (Appendix 1) and 500 µL of 100% ethanol were added. Samples were left at -20°C overnight. The tubes were then centrifuged for 10 minutes at 13,000 rpm, the supernatant tipped off and the pellets washed with 70% ethanol 3 times before air drying. Once air-dried, pellets were re-suspended in 100 µL de-ionised H_2O, left at 4°C for 1 hour and vortexed. Samples were stored at -20°C.

II.3.1.2. *Nick translation reaction*

The probes were made using nick translation (Rigby et al., 1977). This technique involved the use of a dual enzyme: DNase I and DNA polymerase I act in tandem to incorporate labelled nucleotides (dNTPs) into the DNA sequence.

The nick translation reaction mix:

- dH_2O: 7.7 µL
- DNA: 3 µL
- 10x Nick translation buffer: 2 µL
- Non-labelled dNTP: 2 µL
- Labelled dNTP: 0.5 µL
- DNA Polymerase I (10 U/µL): 5 µL
- DNase I (100 mU/µL): 0.8 µL
- Total: 20 µL

Three different labelled dNTPs were used: Chroma Tide Alexa Fluor 488-5-dUTP (Life Technologies™; C11397) (green colour), Chroma Tide Alexa Fluor

594-5-dUTP (Invitogen; C11400) (red colour) and Chroma Tide Alexa Fluor 546-14-dUTP (Life Technologies™; C11401) (gold colour). The tube was incubated for 2h at 16°C in dark conditions.

II.3.1.3. *Ethanol precipitation of the probe*

For probe precipitation, 160 µl of single stranded DNA (SS DNA) working solution (Appendix 1) was added to the 20 µl nick translation probe reaction and vortexed. 500 µl of 3M sodium acetate solution (Appendix 1) was added to the reaction mixture and incubated at -20°C overnight. The tube was then centrifuged at 12,000 rpm for 30 mins at 4°C. The supernatant was discarded and the probe pellet washed with 70% ethanol and centrifuged at 12,000 rpm for 5 minutes two times. The probe was air-dried in the dark for 10 minutes. The pellet was then dissolved in 20 µL of 2xSSC+1xTE buffer (Appendix 1) for 1 hour on ice. The solution was then mixed by tapping on the tube and stored at -20°C.

II.3.2. Preparation of blocking DNA

500 µL of genomic DNA was aliquoted in a 1.5 mL tube, which was sealed tightly with NASCO film. The DNA was treated at 110°C for 10 minutes. The tube was then stored at -20°C.

II.3.3. Preparation of metaphase spreads from root tips

Preparation of metaphase spreads was done according to the methods described in Kato et al. (2004). Seeds were germinated in a Petri dish on filter paper hydrated with fresh water. After a few days, 3 roots were cut off and placed in a 0.5 mL tube, the lid of which had been pierced. The tubes were transferred into a steel chamber filled with nitrous oxide for 2 hours at 10 bar. The roots were then fixed in 90% acetic acid for 10 minutes before being washed three times with fresh water. The roots were then put gently on a filter paper to remove the excess of water. The tip of the root sections (approximately 1 mm) containing the dividing cells, were dissected and digested in 20 µL of enzyme solution (1% pectolyase Y23 and 2% cellulose

Onozuka R-10) (Appendix 1) at 37°C. Digestion time was between 50 and 53 minutes according to the size of the roots and the back cross stage of the plant. After digestion, the root tips were washed three times in 70% ethanol. Root tips were carefully crushed on the tube wall with a dissection needle. The tubes were then centrifuged at 600 rpm for 2 minutes. The ethanol was carefully removed, the pellet briefly dried by keeping the tube with the lid open for 10 minutes and the cells were suspended in 14 µL of 100% acetic acid. After 1 hour, 7 µL of the cell suspension was dropped on to a glass slide placed in a moist box. The box was closed to allow the slides to dry slowly at room temperature for 10 mins. Slides were checked using a microscope and only those carrying at least three good metaphase spreads were conserved for the next step. Slides were stored at -20°C.

II.3.4. *In Situ* Hybridization

Slides were treated twice with UV light at 0,125 Joules in order to cross link the chromatin on the slides. Slides were then probed by using the following mixture (10 µL/slide):

<u>Single colour GISH mixture</u>

- Total wheat blocking gDNA: 4 µL
- Probe gDNA(100 ng/µL): 1 µL
- 2 x SSC in 1 x TE buffer: 5 µL (Appendix 1)

<u>Multi-colour GISH mixture</u>

- B genome blocking gDNA: 4 µL
- A genome probe gDNA (100 ng/µL): 0.8 µL
- D genome probe gDNA (100 ng/µL): 1.1 µL
- Wild relative genome probe gDNA (100 ng/µL): 0.7 µL
- 2 x SSC in 1 x TE buffer: 3.4 µL

This mixture was dropped on the slides where the cell suspension has been dropped. A cover slip was then put on the slide. Slides were incubated at 75°C for 5 minutes, and then transferred into a closed and moist box overnight at 55°C to allow the hybridization. Slides were then washed with 2 x SSC to remove the cover slip. Vectashield mounting medium with DAPI (Appendix 1) was added to the slides and a new cover slip was put onto the slides avoiding air bubbles. The slides were then observed using a 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. Image analysis was carried out using Meta Systems ISIS and Metafer software (Metasystems GmbH, Altlussheim, Germany).

II.4. Genotyping with KASP markers

II.4.1. Primer dilution

For each marker the common, forward and reverse primers (produced by LGC) were combined in the same tube at a 74 x dilution:

- 395 µL dH_2O
- 15 µL forward primer (100 µM)
- 15 µL reverse primer (100 µM)
- 75 µL common primer (100 µM)

II.4.2. PCR

DNA was extracted according to the protocol explained in the section II.2. Samples were loaded into a 96 wells plate. One well was loaded with de-ionised water as a control. DNA of parental lines were loaded in three wells. 5 µL of each sample were transferred four times into a 384 wells plates. The plate was dried for 20 minutes at 80°C. The PCR reaction mix was prepared with the following components (for one well):

- dH_2O: 2.43 µL
- ROX PACE mix: 2.50 µL (Appendix 1)

- 74x Primers: 0.068 µL

5 µL of the PCR reaction mix was added to each well.

PCR plate was run on the ProFlex PCR system (Applied Biosystems) with the following programme:

Step	Temperature (°C)	Time (minutes)	No. of cycles
Denaturation	94.0	15.00	
	94.0	0.25	X 10
Annealing	65.0	1.0	X 10
Elongation	94.0	0.25	X4 0
	57.0	1.0	X 40
Final Hold Point	4.0		

Results were analysed using QuantStudio™ 5 Real-Time PCR System and QuantStudio™ Design and Analysis Software v1.4.3 (Applied Biosystems).

III. Chapter 3 : Test a wide range of relatives species for their ability to restore *T. timopheevii* cytoplasm

Summary

Thirteen different wild species genomes were tested for their ability to restore *T. timopheevii* cytoplasm. Wheat/wild relative amphidiploids were chosen as a way to test the whole genome of the wild species, and thus used as pollinator parent on CMS lines. The low seed set, the high percentage of shrivelled F_1 seeds, the low level of germination and the plant lethality reduced dramatically the number of wild relatives genome tested. We showed that *S. anatolicum* was carrier of restorer factor(s). Real level of fertility restoration was not established because of the unbalanced genome. Moreover, we highlighted the ability of Chinese Spring wheat to restore partially the male fertility in winter wheat background.

III.1. Introduction

III.1.1. CMS based on *T. timopheevii* cytoplasm

III.1.1.1. *T. timopheevii*

T. timopheevii is a tetraploid wheat (2n = 4x = 28, A^tA^tGG) originating from Georgia and domesticated from *T. araraticum* (Jakubz.) (Kilian et al., 2009; Mori et al., 2009). It has both a wild (*T. timopheevii* (Jakubz.) ssp. *armeniacum*) and a cultivated form (*T. timopheevii* (Zhuk.) Zhuk) (van Slageren, 1994). The origins of the two genomes of *T. timopheevii* have been subject to many discussions. *T. monococcum* (Lilienfeld, 1951) and *T. boeoticum* (Konarev et al., 1979) have been suggested to be the donor of the A^t genome, but strong evidence led to the conclusion that the A^t genome originated from *T. urartu* (Kilian et al., 2006). The G genome comes from the S genome of *Ae. speltoides* which is the cytoplasm donor as well (Mori et al., 1997). Two different origins for *T. timopheevii* have been proposed. The first is a monophyletic origin in which *T. timopheevii* and *T. turgidum* derived from a common ancestor and then diverged due to chromosomal rearrangements or introgressive

hybridization with related diploid species (Gill and Chen, 1987). The second hypothesis relies on a diphyletic origin in which *T. timopheevii* and *T. turgidum* resulted from two separate hybridization events (Mori et al., 2009) (**Figure III-1**). This would mean that two different accessions of *Ae. speltoides* were involved.

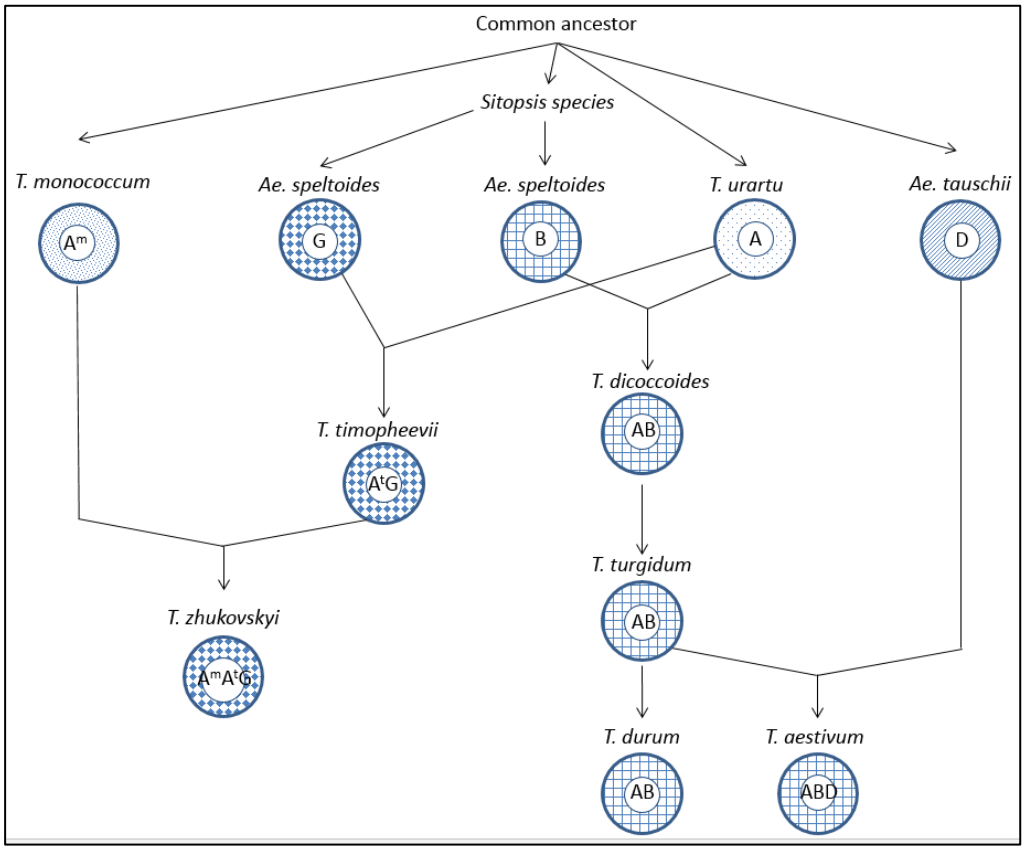


Figure III-1 Diphyletic origin of wheat species

The inner circle represents the nuclear genome. The outer circle represents the cytoplasm genome whom the origin is symbolised by different motif

The *T. timopheevii* genome is a huge reservoir of interesting traits to improve common wheat such as resistance genes for stem rust, leaf rust or powdery mildew (Friebe et al., 1996; Gill et al., 2006). *T. timopheevii* is also a source for tolerance to abiotic stresses, such as drought, and the cultivated form of this species has good bread-making quality with high protein content (Mikó et al., 2015). Many of these genes have already been transferred into the wheat genome (McIntosh et al., 2013). Another interesting aspect of *T. timopheevii* is the ability of its cytoplasm to cause sterility in common wheat.

III.1.1.2. *T. timopheevii cytoplasm causes male fertility in common wheat*

In 1962 Wilson and Ross introduced the nucleus of *T. aestivum* cv. Bison into the cytoplasm of *T. timopheevii* Zhuk. by using *T. timopheevii* Zhuk. as the female line for the initial cross, followed by several back crosses with the cultivar Bison. Complete and stable male sterility was observed in the alloplasmic lines (Wilson and Ross, 1962). After this discovery, *T. timopheevii* cytoplasm has been widely studied and became the most widespread in the CMS hybrid wheat program and is considered as the most reliable source to achieve male sterility (Koekemoer et al., 2001). Indeed the cytoplasm causes complete sterility (Tsunewaki et al., 1996) with very few deleterious effects on the phenotype contrary to all other *Aegilops* or *Triticum* cytoplasms (Tsunewaki et al., 1976; Wilson and Driscoll, 1983). *T. timopheevii* cytoplasm causes a slight seed germination reduction, has a slight negative effect on some vegetative characters, a slight increase in the number of spikelets but no delay in the heading date or reduction of plant height (Tsunewaki et al., 2002). The cytoplasm of *T. timopheevii* also causes pre-harvest sprouting (Clayton and Ellis, 1976; Doig et al., 1975; Jönsson, 1976) and increases in kernel shrivelling (Clayton and Ellis, 1976) and protein content and alpha-amylase activity (Miller et al., 1975). The male sterile phenotype is characterized by the production of shrivelled and curved anthers which carry unviable pollen and are borne on filaments that do not elongate (Wilson and Ross, 1962). It has also been shown that the expression of the sterility in common wheat carrying *T. timopheevii* cytoplasm is dependant on the environment, but this phenomenon is still not clearly understood (Lucken and Maan, 1967; Wilson, 1968a).

Studies of the mitochondrial DNA (mtDNA) in the *coxI* region of *T. aestivum* (with *T. aestivum* cytoplasm), *T. timopheevii* (with *T. timopheevii* cytoplasm), a CMS line and a restorer line (*T. aestivum* nucleus and *T. timopheevii* cytoplasm) showed differences. A chimeric gene, named as *open reading frame 256 (orf256)*, residing upstream to *coxI*, is present in *T. timopheevii* mtDNA but not in *T. aestivum* mtDNA (Rathburn and Hedgcoth, 1991; Rathburn et al., 1993).

The -1 to -228 5’ flanking sequence of *orf256* in *T. timopheevii* is identical to the analogous region from *coxI* of *T. aestivum*. The first eleven amino acids encoding *orf256* are identical to *coxI*. The *orf256* is a combination of 261 bp of the *coxI* gene region (228 bp of 5’ flanking region and 33 bp of encoded N-terminus) and a segment of DNA from another origin (Rathburn et al., 1993). The nucleotide sequences of the *orf256* region are identical in *T. timopheevii*, CMS lines and restorer lines. However, the transcription is altered by the nuclear background. In *T. timopheevii, orf256* transcripts are only synthesized to a very low level or are not stable, whereas CMS and restorer lines (*T. aestivum* nucleus and *T. timopheevii* cytoplasm) produce a co-transcript of *orf256* and *coxI* (Song and Hedgcoth, 1994a). In CMS lines, *orf256* is transcribed together with *coxI* and expresses a 7 kDa protein, which is a protein of the inner membrane of the mitochondria (Song and Hedgcoth, 1994b). By introducing restorer genes into CMS lines, translation of the co-transcripts is disrupted and only the cotranscript COXI is synthetized (Song and Hedgcoth, 1994b). This 7 kDa protein is not detected in the restorer line. Nevertheless, the role of the 7 kDa protein remains unknown. Interestingly, a CMS line with the cytoplasm of *Ae. speltoides* produces the 7 kDa protein from *orf256* (Song and Hedgcoth, 1994b), which supports the study of Mori et al., (1997), leading to the conclusion that *Ae. speltoides* is the cytoplasm donor of *T. timopheevii*. The presence and organization of *orf256*-like in wheat progenitors, wheat relatives and more distant cereals has been studied (Hedgcoth et al., 2002). Various elements of the *orf256* sequence have been detected in barley, rye, *Ae. bicornis*, *Ae. searsii*, *Ae. sharonensis*, *Ae. speltoides, Ae. tauschii* and *T. monococcum*. This sequence is not present in *T. turgidum*. However, only *T. timopheevii* and *Ae. speltoides* express *orf256* as a RNA product but not the corresponding protein product (Hedgcoth et al., 2002). Although *Ae. speltoides* is considered as the cytoplasm donor of *T. turgidum* and *T. aestivum* (Kilian et al., 2006) the mtDNA has diverged from each other in the region immediately upstream to *coxI*. It has been shown that the *orf256-coxI* RNA binds only with two mitochondrial proteins in *T. aestivum*, a 39 kDa and a 42 kDa protein,

which could be involved in the elimination of the *orf256* sequence from *T. aestivum* mtDNA (El-Shehawi et al., 2003).

III.1.1.3. *Fertility restoration of T. timopheevii cytoplasm*

In order to develop a complete CMS system, male fertility has to be totally restored in the F_1 hybrid. The first source of *Restorer of fertility* (*Rf*) genes is *T. timopheevii* itself (Livers, 1964). However, the level of the restoration is not total and is strongly dependant on the environment which reduces the utilization of these restorers as the quality of hybrid wheat products is impaired (Johnson and Patterson, 1973; Wilson and Driscoll, 1983). Additionally, unpollinated florets of male-sterile wheat lines have shown high susceptibility to infections with ergot (*Claviceps purpurea*) (Mantle and Swan, 1995). Prospecting for total and stable restorers has been done in the near relatives of *T. timopheevii*. Some common wheat genotypes have shown the ability to restore *T. timopheevii* cytoplasm, the fertility of the F_1 varying from a few seeds at the base of the spike to nearly complete seed set (Wilson and Driscoll, 1983). Monosomic analysis has been used to locate *Rf* genes on lines derived from *T. timopheevii*. In total seven *Rf* genes have been found on the wheat genome: *Rf1* on chromosome 1A (Bahl and Maan, 1973; Du et al., 1991; Robertson and Curtis, 1967; Yen et al., 1969), *Rf2* on chromosome 7D (Bahl and Maan, 1973; Yen et al., 1969), *Rf3* on chromosome 1B (Bahl and Maan, 1973) and present as well on chromosome 1B of *T. spelta* L. var. *duhamelianum* (Kihara, 1967b; Tahir and Tsunewaki, 1969), *Rf4* on chromosome 6B (Bahl and Maan, 1973; Maan et al., 1984; Yen et al., 1969), *Rf5* on chromosome 6D (Yen et al., 1969), *Rf6* on chromosome 5D (Bahl and Maan, 1973) and *Rf7* on chromosome 7B (Bahl and Maan, 1973; Maan et al., 1984). Recently, a major locus on chromosome 2D has been proposed as *Rf8* (Sinha et al., 2013) (**Figure III-2**).

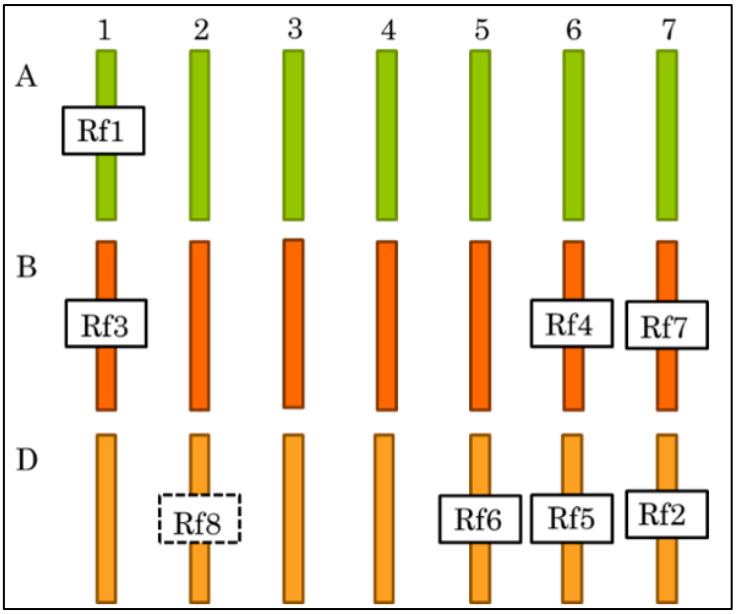


Figure III-2 Localization of the restorer genes in common wheat genome against *Triticum timopheevii* cytoplasm

A, B, D indicates the different wheat genome, the number 1 to 7 indicate the chromosome number

The ideal restoration line carries a single dominant restoration gene leading to total and stable fertility restoration (Sage, 1972). A single gene restoration has been reported leading to a high level of restoration (Hughes and Bodden, 1977). However, most results suggest that total restoration of fertility is due to at least three genes functioning as cumulative dominant genes (Sage, 1972; Wilson, 1968b) (**Table III-1**). In order to overcome the partial sterility in a CMS hybrid, different restorer genes can be combined (Johnson and Patterson, 1977) but as the *Rf* genes are located on chromosomes of different groups (1, 2, 5, 6 and 7), the complexity of pyramiding *Rf* genes in any cultivar is increased (Sage, 1976). Different genetic architectures of fertility restoration have been found in different restorer lines. One of the most famous restorer cultivars is Primepi, a winter wheat variety registered in 1962 in France. The restoration was first considered to be controlled by a single monogenic gene (Goujon and Ingold, 1967). This hypothesis was confirmed by Geyer et al., (2016). However, in other studies it appeared that restoration is under a digenic dominant control involving chromosomes 1B (*Rf3*) and 5D (*Rf6*) (Bahl and Maan, 1973; Miller and Schmidt, 1970; Miller et al., 1974) but with *Rf3* showing a higher

expressivity than *Rf6*. In the line R113, *Rf1* interacts with *Rf4*, but the effect of these two loci are not additive (Geyer et al., 2018). The only case of total restoration with a single restorer gene has been observed in *T. spelta* L. var. *duhamelianum* involving chromosome 1B (Tahir and Tsunewaki, 1969).

Table III-1 Main wheat restorer lines against *T. timopheevii* cytoplasm

Restorer line	***Rf* genes**	**References**
R1-Lee	*Rf1*, *Rf2*	(Bahl and Maan, 1973)
R2-Sonora 64	*Rf1*, *Rf2*, *Rf4*	(Bahl and Maan, 1973)
R3	*Rf1*, *Rf2*	(Bahl and Maan, 1973)
Marquis	*Rf1*, *Rf2*	(Robertson and Curtis, 1967)
R4-Dirck	*Rf1*, *Rf2*	(Bahl and Maan, 1973)
R5	*Rf1*, *Rf2*, *Rf7*	(Bahl and Maan, 1973)
Primepi	*Rf3*, *Rf6*	(Bahl and Maan, 1973; Ma and Sorrells, 1995)
R113	*Rf1*, *Rf4*	(Ma and Sorrells, 1995; Maan et al., 1984)
R18	*Rf3*	(Zhou et al., 2005)
R9034	*Rf3*	(Zhou et al., 2005)
R17127	*Rf1*, *Rf6*	(Gotsova et al., 1987)
PWR4099	*Rf8*	(Sinha et al., 2013)
L19	*Rf1*	(Maan, 1985)
T. spelta L. var. *duhamelianum*	*Rf3*	(Ahmed et al., 2001; Tahir and Tsunewaki, 1969)

In addition to these major *Rf* genes, *Rf* genes with small effect, named as modifier loci and including inhibitor loci, have been found. Modifier loci act only in the presence of major *Rf* genes and have low penetrance. The restorer allele of a modifier gene can be carried by the female line which will influence the level of fertility restoration. Different modifier loci are detected depending both on the restorer accessions studied and the CMS lines. For example additional loci have been identified on chromosomes 1B, 2A, 3D, 6A and 6B in the offspring of CMS-Bison crossed by R3 (Robertson and Curtis, 1967) and Chinese Spring (Robertson and Curtis, 1967) and chromosomes 4A, 5A and 5B in the offspring of CMS-Chris crossed by R3 (Tahir and Tsunewaki, 1969). By

crossing CMS-Chris with R113, modifier loci were shown to be located on chromosomes 1B, 2D, 4B, 5A, 5B and 6A (Maan et al., 1984). By crossing CMS-Chris with L19, a descendant of R113 carrying only *Rf1*, chromosomes 2A, 2B, 3A, 3D, 4B, 5B, 6A, 6B and 6D were shown to carry modifier loci (Du et al., 1991). When using Sperber as a CMS line, *Rf1* was found to be linked to a modifier locus on 1BS (Geyer et al., 2018). Inhibitor genes have also been detected on chromosomes 5A, 5B and 6A of R113 (Maan et al., 1984) and 7D of *T. spelta* L. var. *duhamelianum* (Tahir and Tsunewaki, 1969). Despite *Rf3* being a major restorer locus, complete restoration cannot be achieved without the presence of modifier loci (Würschum et al., 2017b). Thus a joint selection has to be done for major restorer genes and modifier genes to reach complete fertility.

The genetic background is also an important factor in the inheritance of the fertility restoration (Maan et al., 1984; Virmani and Edwards, 1983; Wilson and Driscoll, 1983). Male sterile genotypes can be classified as “easy to restore” or “difficult to restore” (Trupp, 1976; Wilson, 1968b). In some cases, despite the presence of a restorer allele, the line does not show any restoration of fertility (Maan, 1985; Maan et al., 1984) as the expression of the fertility is strongly dependant on the environment (Bahl and Maan, 1973; Johnson et al., 1967; Sage, 1972; Virmani and Edwards, 1983; Wilson, 1968b).

Development of molecular markers to track *Rf* genes is essential to optimize their use in hybrid breeding programs. However, up to now, very few markers haven been developed for that purpose and not all the *Rf* genes have been studied (**Table III-2**).

Table III-2 Molecular markers developed to track the different restorer genes in common wheat genome

Rf gene	Chr.	Marker name	Wheat line	Type of markers	Reference
Rf1	1AS	*Xgwm136*	7269-10	SSR	(Zhang et al., 2003)
Rf1	1AS	*Xgwm136* *Xgpw7062* *Xgdm33*		SSR	(Li et al., 2014)
Rf3	1BS	*Xbcd249* *Xcdo442*	Primepi	RFLP	(Ma and Sorrells, 1995)
Rf3	1BS	*XksuG9c*	*T. spelta* L. var. *duhamelianum*	RFLP	(Ahmed et al., 2001)
Rf3	1BS	*Xgwm550*	7269-10	SSR	(Zhang et al., 2003)
Rf3	1BS	*Xbarc207* *Xgwm131* *Xbarc61*	R18 R9034	SSR	(Zhou et al., 2005)
Rf3	1BS	*IWB72107*	Primepi	SNP	(Geyer et al., 2016)
Rf4	6BS	*Xksug48*	R113	RFLP	(Ma and Sorrells, 1995)
Rf4	6BS	*Xgpw1079* *Xgwm193* *Xgpw7011* *Xgwm508*	R113	SSR	(Li et al., 2014)
Rf6	5D	*Xcdo786*	Primepi	RFLP	(Ma and Sorrells, 1995)
Rf8	2D	*Xwmc503*	PWR4099	SSR	(Sinha et al., 2013)

However, these markers were not suitable for marker assisted selection of the restorer genes. In the case of *Rf1,* SNPs have been developed to define an haplotype for the genomic selection around *Rf1* (Geyer et al., 2018). This work also allowed confirmation that the *Rf1* restoring allele in R3, R113 and L19 was introgressed from *T. timopheevii*. An informative SNP for *Rf3*, IWB72107, has been developed, leading to the conclusion that almost 10% of the European

common wheat genotypes and 70% of the spelt cultivars carry restorer alleles of *Rf3* (Geyer et al., 2016). In parallel three SNP markers were converted into KASP markers to enable high-throughput screening (Würschum et al., 2017b).

Group 1 chromosomes appear to be a major source of restorer genes for *T. timopheevii* cytoplasm: *Rf1* has been located on 1A and *Rf3* on 1B (Bahl and Maan, 1973). Chromosome 1BS is also involved in the restoration of *Ae. kotschyi* cytoplasm (*Rfv1*) (Mukai and Tsunewaki, 1979), *Ae. mutica* cytoplasm (*Rfm1*) (Tsujimoto and Tsunewaki, 1984b) and *Ae. uniaristata* cytoplasm (*Rfn1*) (Mukai, 1983). Thus, the existence of more effective restoring alleles on chromosome 1G of *T. timopheevii* is very high. The only barrier to the transfer of these alleles into common wheat is the low pairing level between the G genome of *T. timopheevii* and the B genome of *T. aestivum* (Wilson and Driscoll, 1983). Lines produced until now have been derived from a limited number of crosses and thus introgression events (Wilson, 1968a). Higher success might be reached by using for example *ph1* mutant wheat.

Restorer genes against *T. timopheevii* cytoplasm have also been located in other species. Chromosome 6U of *Ae. umbellulata* has been found to carry a *Rf* gene causing partial restoration (Kurauchi et al., 1977). This restorer gene, named as *Rf6*, has been transferred into chromosome 6AS and 6BS of common wheat (Ma et al., 1991, 1995). Rye has been shown capable of restoring *T. timopheevii* cytoplasm (Porter and Tuleen, 1972). Two *Rf* genes have been localized on the rye genome: *Rfc3* on chromosome 6RL and *Rfc4* on chromosome 4RL. Whereas *Rfc3* completely restores fertility, *Rfc4* causes only a partial restoration (Curtis and Lukaszewski, 1993).

Until now no single dominant restorer gene giving a total and stable fertility restoration has been discovered and transferred into common wheat. Other wild relatives might be potential sources of restorers for *T. timopheevii* cytoplasm but they have not be studied because of the various barriers to produce viable interspecific hybrids.

III.1.2. Amphidiploid, a source of genetic variation

The results of an interspecific or intergeneric cross is a F_1 hybrid containing one set of homologous chromosomes of each parent. This hybrid, named amphihaploid, is mostly sterile because of aberrations occurring during meiosis leading to sterile gametes with incomplete chromosome numbers. In order to restore the fertility, chromosome doubling is necessary (Maan and Sasakuma, 1977). The result is an amphidiploid (allopolyploid) carrying total homologous pairs of chromosomes and thus having a normal chromosome behaviour at meiosis (Liu et al., 2014; Nemeth et al., 2015). **Figure III-3** illustrated the production of a fertile wheat/rye amphidiploid.

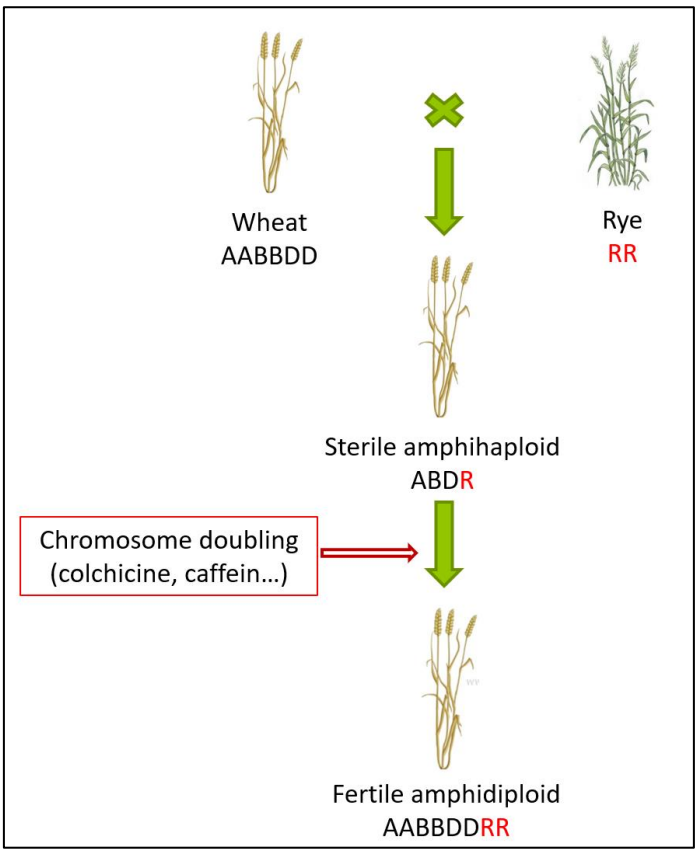


Figure III-3 Crossing scheme for the production of fertile wheat/rye amphidiploid after chromosome doubling

Chromosome doubling is achieved by using an antimitotic agent (Dhooghe et al., 2011). Most of these agents are metaphase inhibitors, leading to the non-separation of chromosomes and thus to the formation of cells with a double set of chromosomes. The most common agent used is colchicine, an alkaloid,

extracted from the wild meadow saffron, *Colchicum automnale*. Colchicine inhibits microtubule polymerization by binding to tubulin and thus preventing the migration of chromosomes/chromatids during anaphase. Colchicine, however, is highly toxic for F_1 interspecific hybrids and also humans (Dhooghe et al., 2011; Würschum et al., 2012). For that reason other drugs have been studied. Herbicides, such as oryzalin, trifluralin or pronamides. affect mitosis. They can be used at lower concentrations compared to colchicine and are less toxic for humans (Dhooghe et al., 2011; Liu et al., 2014). Caffeine, extracted from coffee bean (*Coffea arabica*) has also been proposed as an alternative. Caffeine inhibits cytokinesis, inducing formation of binucleate cells. Caffeine is less efficient than colchicine but less toxic as well for plants and humans (Thomas et al., 1997). One example of a successful synthetic amphidiploid is Triticale (x*Triticosecale* Wittmack) resulting from the cross between wheat and rye followed by colchicine treatment (Lorenz and Pomeranz, 1974). Triticale can be hexaploid (2n=42, AABBRR), resulting from the cross of tetraploid wheat and rye or octoploid (2n=56, AABBDDRR), resulting from the combination between hexaploid wheat and rye.

Spontaneous chromosome doubling can happen in the F_1 amphihaploid, leading to partial fertility. Spontaneous chromosome doubling results from the union of unreduced gametes (Liu et al., 2014; Sattler et al., 2016). Unreduced gametes are the result of meiotic nuclear restitution, a meiotic failure which can happen either at meiosis I or meiosis II, the first division restitution (FDR) and the second division restitution (SDR) respectively (Sattler et al., 2016). FDR includes the absence of chromosome pairing or non-segregation of homologous chromosomes in anaphase I. The unreduced gametes will possess two non-sister chromatids. The nucleus is restored in the first meiotic division and the second division is normal (equational) (Silkova et al., 2011a). FDR has been reported in different wheat wild relative hybrids (Xu and Joppa, 1995). In the case of SDR, the first meiosis is normal but sister chromatids do not segregate in anaphase II. Gametes will thus carry two sister chromatids. These gametes are expected to have an unbalanced chromosome constitution which

might lead to sterility (Liu et al., 2014; Sattler et al., 2016). Some authors have also reported single division meiosis (SDM), also called “mitotic-like division” (Matsuoka and Nasuda, 2004; Zhang et al., 2008, 2007). In such cases, chromosomes behave similarly to mitosis: chromosomes are separated into chromatids at anaphase I without being followed by a second division, leading to the formation of dyads exactly as for FDR (Zhang et al., 2008, 2007). Bread wheat is a perfect example of a natural amphidiploid, the result of two hybridization events followed by duplication to lead to a fertile hexaploid species (Matsuoka et al., 2013).

III.1.3. Aim and objectives of the chapter

The main aim of this chapter is to screen the ability of a wide range of wild relatives to restore *T. timopheevii* cytoplasm by using amphidiploids. One approach to find new restorer genes against *T. timopheevii* cytoplasm is to use wheat – wild relative amphidiploids, as it is the easiest way to test the whole genome of the wild species.

III.2. Material and Methods

III.2.1. Plant material

Four different hexaploid wheat varieties carrying *T. timopheevii* cytoplasm were used as the female parent. Two lines were spring type and two lines were winter type. The CMS lines were provided by Limagrain and were at the BC_3 generation. The male parents were a selection of the 30 amphidiploids produced at the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) (Nemeth et al., 2015) (**Table III-3**). These amphidiploids were the result of crosses between 13 accessions of different wild species (from genera *Aegilops*, *Secale*, *Thinopyrum* and *Triticum*) with different spring wheat genotypes (Chinese Spring (CS), Chinese Spring *ph1* mutant, Highbury, Paragon and Pavon 76) leading to 30 different amphidiploids. The production of these amphidiploids has been described by Nemeth et al., (2015).

Table III-3 Amphidiploids used as pollen donor

Amphidiploid	Wheat parent	Wild relatives accession	Wild genome
76/12/4	Paragon	*Ae. caudata 2090001*	C
73/5/1	Highbury	*Ae. caudata 2090002*	C
79/1/38	Paragon	*Ae. comosa 276970*	M
28/1/78	CS	*Ae. mutica 2130004*	T
29/1/18	CS	*Ae. mutica 2130008*	T
24/5/18	Highbury	*Ae. mutica 2130012*	T
27/1/21	CS	*Ae. mutica 2130012*	T
31/1/1	Pavon 76	*Ae. mutica 2130012*	T
51/1/3	Paragon	*Ae. mutica 2130012*	T
38/4/10	Highbury	*Ae. speltoides 2140008*	S
40/4/32	CS	*Ae. speltoides 2140008*	S
43/1/20	Pavon 76	*Ae. speltoides 2140008*	S
44/1/3	Pavon 76	*Ae. speltoides 449340*	S
32/1/17	Pavon 76	*Ae. umbellulata 542377*	U
33/1/12	CS	*Ae. umbellulata 554410*	U
34/1/7	CS	*Ae. umbellulata 554410*	U
1/6/2	CS	*S. anatolicum P208/141*	R
94/1	Pavon 76	*S. anatolicum P208/141*	R
5/8/7	CS	*S. anatolicum P208/142*	R
95/6/4	Highbury	*S. anatolicum P208/142*	R
99/11/8	CS	*S. anatolicum P208/142*	R
92/1/9	Pavon 76	*S. iranicum P208/11*	R
90/4/6	CS	*S. vavilovii 573649*	R
85/1/4	CS	*Th. bessarabicum 531711*	J
23/1/26	CS mutant	*Th. bessarabicum 531712*	J
49/7/5	CS mutant	*Th. bessarabicum 531712*	J
48/1/15	CS mutant	*Th. elongatum 401007*	EeEbExStSt
81/22/8	CS	*Th. turcicum P208/201*	EEEEE
35/2/6	CS	*T. timopheevii P95-99-1-1*	A^tG
82/1	Highbury	*T. turgidum* ssp. *carthlicum 94748*	AB

III.2.2. Crossing program

The crossing scheme is presented in **Figure III-4**. As CMS lines were used as female lines there was no need for emasculation. Each head of the CMS line was covered with a bag before the opening of the spikelets to avoid pollen

contamination. Once the stigma was fluffy and receptive, heads were pollinated with amphidiploid pollen. After pollination, heads were bagged to protect them. Each head was tagged with the parental pedigree.

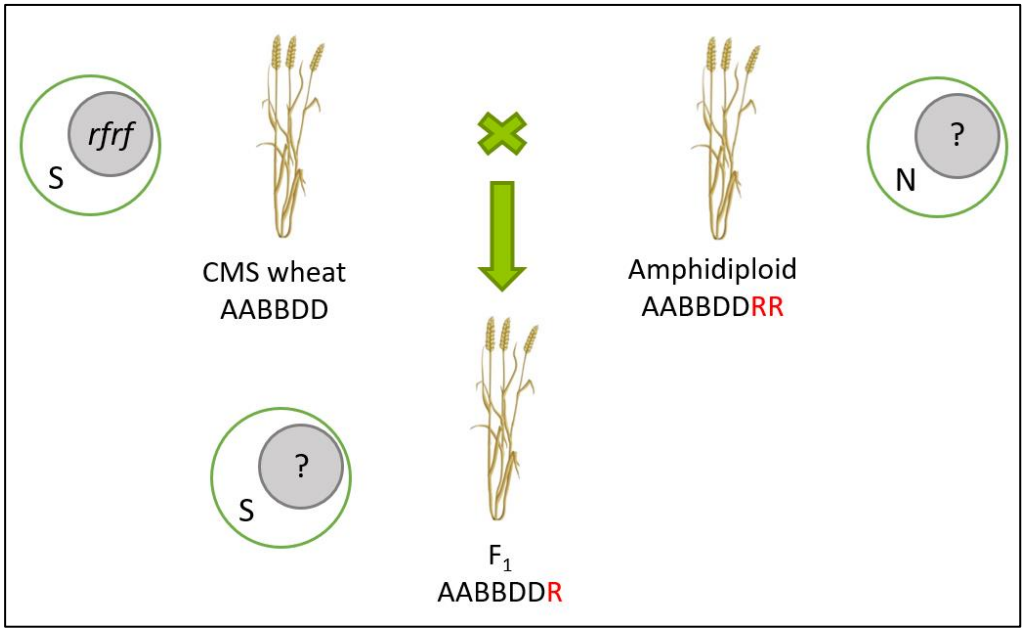


Figure III-4 Crossing scheme of the production of CMS x wheat/wild relatives (rye) amphidiploid F_1

The cell of each line is represented by the cytoplasm (outer circle) and the nucleus (inner circle). "S" means sterile cytoplasm, "N" normal cytoplasm, "*rf*" non-restorer allele, "?" indicates the absence of information about the presence of a restorer allele in the wild genome

In parallel, various crosses were carried out as controls. In order to check the total male sterility of the CMS line, one head of each plant was self-fertilised. Moreover, in order to study the influence of *T. timopheevii* cytoplasm, normal wheat genotypes were used as female parents and pollinated with the pollen of the amphidiploids. Finally pollen from the wheat used to produce the amphidiploids (Chinese Spring, Chinese Spring *ph1* mutant, Highbury, Paragon and Pavon 76) was used to pollinate the CMS line to check the absence of restorer factors in their genomes against *T. timopheevii* cytoplasm.

III.2.3. F_1 germination

Germination of the F_1 seeds was carried out according to the protocol (refer to Chapter II.1.1). As most of the seed was shrivelled, embryo culture was used

to enhance the germination success. The seeds were sterilised with 1 mL 5% ClNaO (10% solution-sigma Aldrich, Appendix 1), the embryo was rescued and placed on a culture medium (MS + 2% sucrose + 1% agarose, Appendix 1) under sterile conditions. Once the seed germinated, it was sown into soil (John Innes compost No. 2). F_1 plants generated with CMS winter wheat were put into vernalisation for eight weeks (at 8°C with 12 hours light per day), F_1 plants generated with CMS spring wheat for four weeks. Plants were grown in the glasshouse (at 21°C, 16 hours light per day).

III.2.4. Assessment of the success of the crosses

Various observation was done to determine the success of the crosses:

- Average number of seeds per head = (Total number of seeds)/(Total number of heads)
- Percentage of fertile heads = (Number of heads setting seeds)/(Total number of heads) *100
- Percentage of shrivelled seeds = (Number of shrivelled seeds)/(Total number of seeds) *100
- Percentage of germination = (Number of seeds germinated)/(Total number of seeds sown) *100
- Percentage of viable plants = (Number of adult plants)/(Number of seeds germinated) *100

III.2.5. Assessment of fertility restoration

To determine the male fertility restoration, heads of the F_1 plants were bagged prior to anthesis and harvested after ripening. On these spikes the number of seeds as well as the number of spikelets was counted and both were averaged per plant. The seed set was calculated according to the formula:

- Fertility for one spike = Number of seeds/Number of spikelets

A plant without any self-fertilised seed was considered as male sterile, whereas a plant with at least one seed was considered as male fertile.

III.3. Results

III.3.1. Cross results

A total of 1229 crosses were done producing 1041 F_1 seeds, an average of 0.8 seeds/head. In total 20.7% of heads pollinated set seeds. **Table III-4** shows a high variation in seed set and the percentage of fertile head in relation with the wild species tested through the amphidiploids. The crosses done with *Ae. caudata*, *S. anatolicum*, *T. turgidum* and *Th. turcicum* amphidiploids were the most successful with 1.9, 1.2, 3.9 and 4.1 seeds/head respectively. The heads pollinated by these amphidiploid set seeds of 30.4%, 27.4%, 51.6%, 23.1% and 48.8% respectively. In the case of the *S. vavilovii* amphidiploid, no seed was obtained from the 11 crosses done.

Table III-4 Seed set and head fertility for each wild species

Wild species	Number of seed/heads	Percentage fertile head
Ae. caudata	1.9 (107/56)	30.4% (17/56)
Ae. comosa	0.3 (10/35)	17.1% (6/35)
Ae. mutica	0.6 (175/270)	21.1% (57/270)
Ae. speltoides	0.4 (86/233)	15.0% (35/233)
Ae. umbellulata	0.4 (40/93)	15.1% (14/93)
S. anatolicum	1.2 (217/179)	27.4% (46/179)
S. iranicum	0.5 (40/82)	15.9% (13/82)
S. vavilovii	0.0 (0/11)	0.0% (0/11)
T. timopheevii	0.1 (9/62)	9.7% (6/62)
T. turgidum	3.9 (122/31)	51.6% (16/31)
Th. bessarabicum	0.7 (64/91)	23.1% (21/64)
Th. elongatum	0.1 (1/45)	2.2% (1/45)
Th. turcicum	4.1 (170/41)	48.8% (20/41)
Total	0.8 (1041/1229)	20.7% (255/1229)

Within the same wild species a variation of the seed set was observed depending on the amphidiploid used. **Figure III-5** showed the average number of seeds produced for each of the 30 amphidiploids used as pollen donor. For

example, in the case of *Ae. caudata*, 0.3 seeds/head were obtained with the pollen of amphidiploid 73/5/1 and 3.0 seeds/head for amphidiploid 76/12/4. In the case of *Ae. mutica* amphidiploids, the lowest seeds set was obtained with the amphidiploid 24/5/18 (0.1 seeds/head) and the highest with the amphidiploid 51/1/3 (2.1 seeds/head).

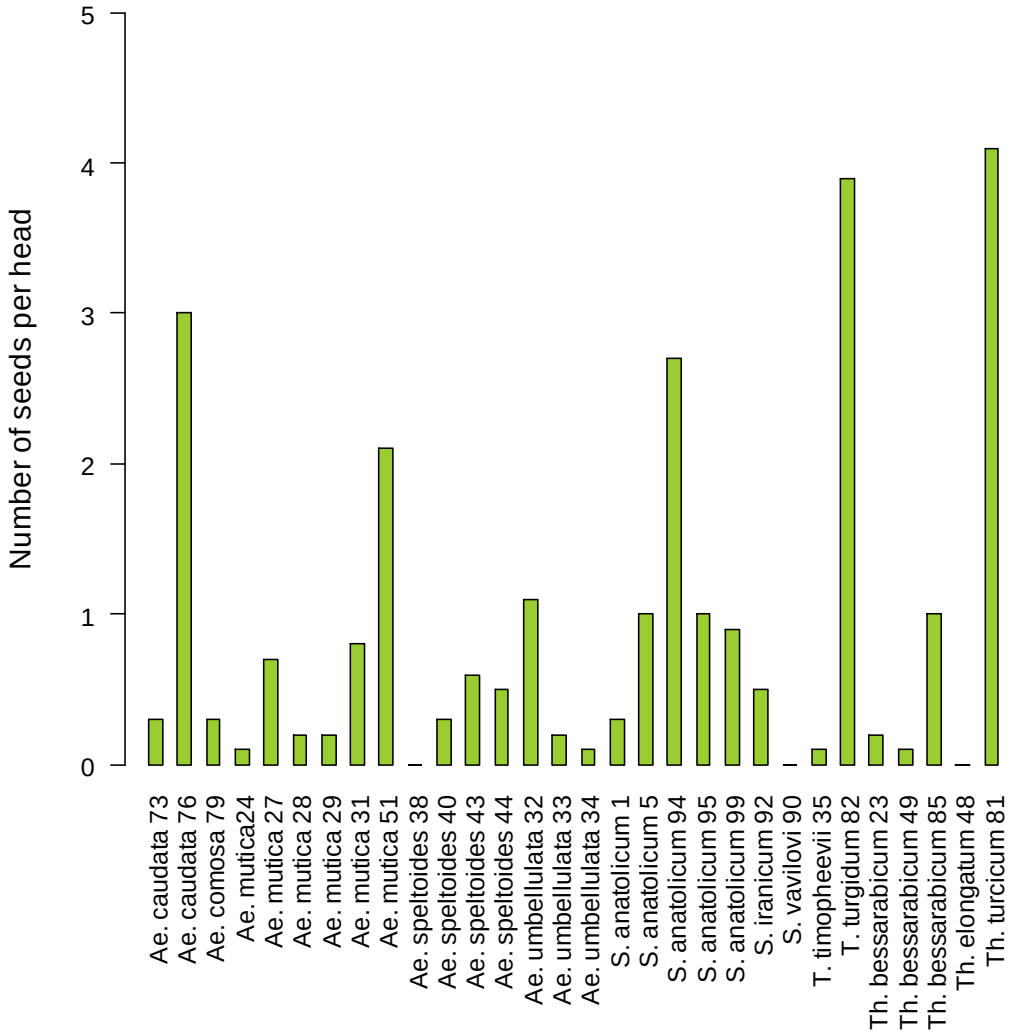


Figure III-5 Average number of seeds per head set on the CMS lines obtained with each amphidiploid

Seed set variation was also observed depending on the CMS genotype used as the female parent (**Figure III-6**). In the case of the spring wheat varieties, between 1.4 and 1.6 seeds/head were produced, whereas for the winter wheat varieties, 0.2 to 0.6 seeds/head were produced.

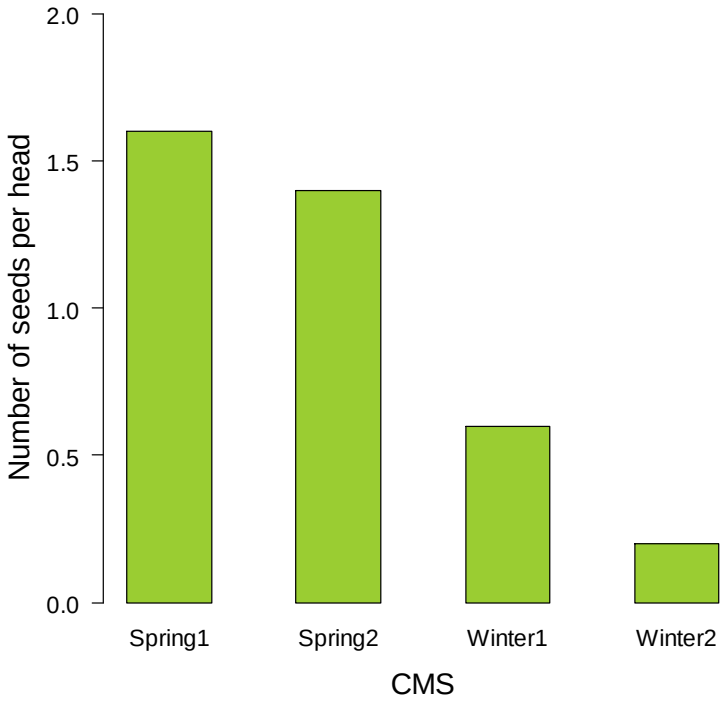


Figure III-6 Average number of seeds per head for each CMS genotype

III.3.2. F_1 seed shape

The evaluation of the shape of F_1 seeds was carried out, as it is considered to be a good indicator of the success of the crosses (**Figure III-7** and **Figure III-8**). Crosses done with some amphidiploids resulted in F_1 seeds that were completely shrivelled such as with *Ae. caudata* amphidiploid 73/5/1 and *Ae. mutica* amphidiploids 24/5/18 and 28/1/78. Other amphidiploids used as pollen donors led to a high percentage of shrivelled F_1 seeds (over 90%), e.g. *Ae. caudata* amphidiploid 76/12/4 (97% of F_1 seeds were shrivelled) and *Ae. mutica* amphidiploids 27/1/27 (97%), 31/1/1 (91%) and 51/1/3 (97%). In the case of *Ae. mutica* amphidiploid 29/1/18 and the *S. anatolicum* amphidiploid 94/1, only 50% of the progeny was shrivelled. The percentage of shrivelled seeds was lower when the following amphidiploids were used as pollen donor: *Th. bessarabicum* amphidiploid 85/1/4 (34%), *T. turgidum* amphidiploid 82/1 (11%) and *Th. turcicum* amphidiploid 81/22/8 (6%). Finally out of the 4 F_1 seeds produced with *Ae. umbellulata* amphidiploid 33/1/12 as male parent, none were shrivelled.

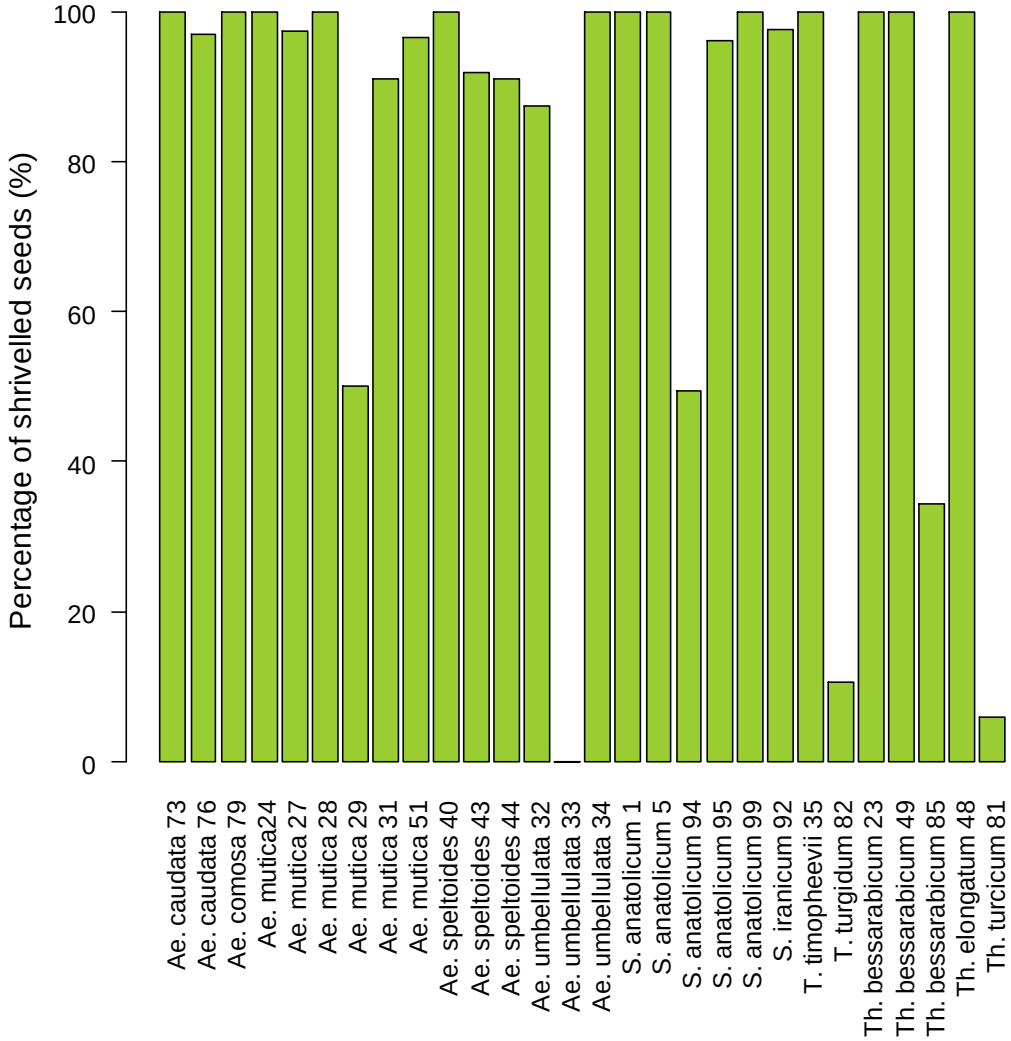


Figure III-7 Percentage of shrivelled F_1 seeds obtained with each amphidiploid

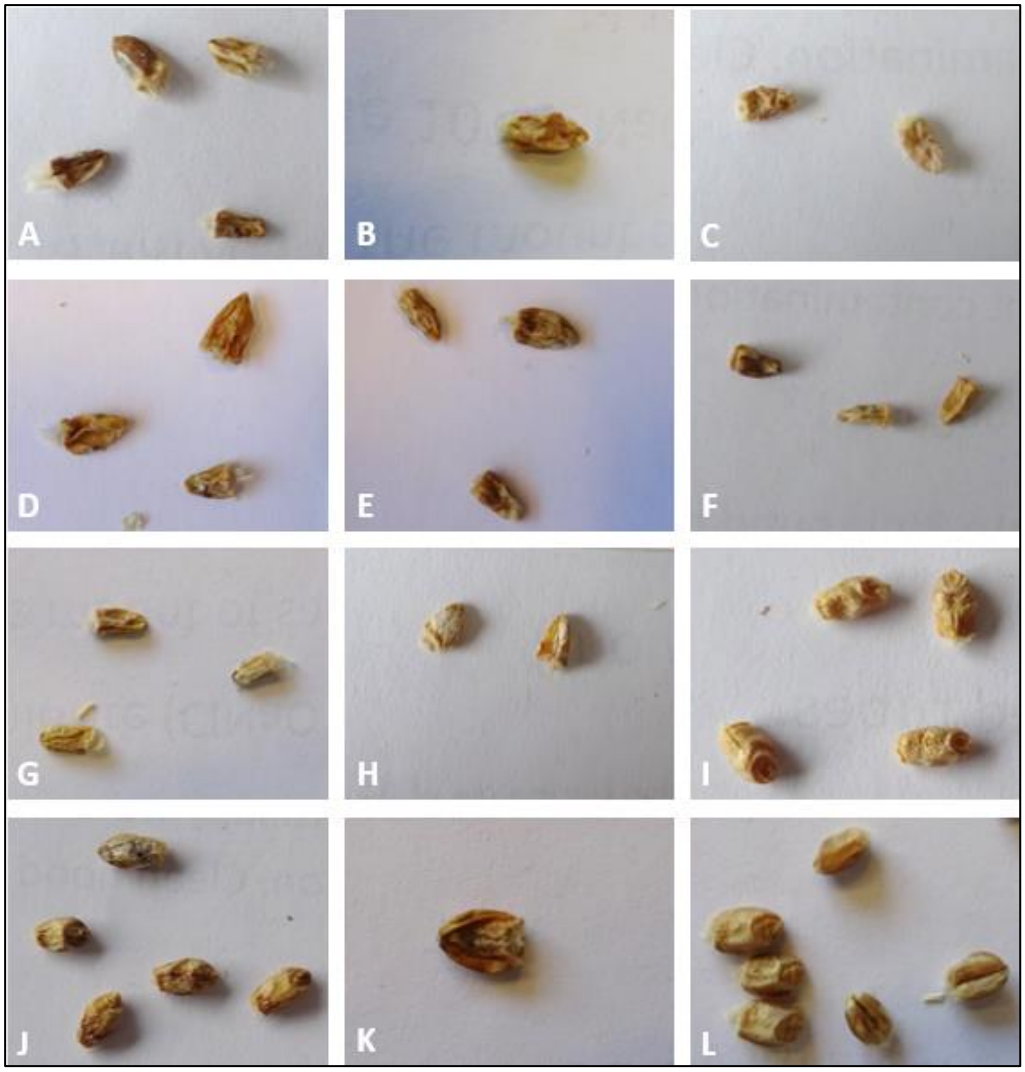


Figure III-8 F_1 seeds shape resulting from the cross between CMS wheat and amphidiploids

A) Amphidiploid *Ae caudata* 76, B) Amphidiploid *Ae. comosa* 79, C) Amphidiploid *Ae. mutica* 31, D) Amphidiploid *Ae. speltoides* 44, E) Amphidiploid *Ae. umbellulata* 32, F) Amphidiploid *S. anatolicum* 95, G) Amphidiploid *S. iranicum* 92, H) Amphidiploid *T. timopheevii* 35, I) Amphidiploid *T. turgidum* 82, J) Amphidiploid *Th. bessarabicum* 85, K) Amphidiploid *Th. elongatum* 48, L) Amphidiploid *Th. turcicum* 81.

III.3.3. Germination rate and viable plants

Out of the 1041 F_1 seeds produced, a total of 428 F_1 seeds were germinated, followed in some cases by embryo rescue. 149 F_1 seeds germinated and only 116 F_1 plants reached the adult stage and headed (**Figure III-9** and **Table III-5**). Seeds from any different crosses did not germinate, e.g. seeds from crosses between CMS and *Ae. caudata* amphidiploid 73/5/1, *Ae. comosa* amphidiploid 79/1/38 and *Ae. mutica* amphidiploids 24/5/48, 27/1/21, 28/1/78 and

29/1/18. Only 5% of the F_1 seeds sown from *Ae. caudata* amphidiploid 76/4/12 germinated (2 seeds out of 40 sown) but none of them reached the adult stage. The F_1 seeds from *Ae. mutica* amphidiploids 31/1/1 and 51/1/3 showed a 67% and 50% germination rate respectively, but only 3 F_1 reached the adult stage. The only F_1 seed from *Ae. umbellulata* amphidiploid 33/1/12 which germinated died during vegetative growth. F_1 seeds from four different *S. anatolicum* amphidiploids, 1/6/2, 94/1, 95/6/4 and 99/11/8, germinated at the rate of 25%, 76%, 8% and 13% respectively but only F_1 seeds of the first two amphidiploids grew to maturity (100% and 100% respectively). 75% of the F_1 seeds from the *T. turgidum* amphidiploid germinated with 97% reaching maturity. For *Th. bessarabicum* amphidiploids, only F_1s from 23/1/23 (100%) and 85/1/85 (59%) germinated, with 67% and 76% respectively reaching the heading stage. Finally, 67% of the F_1 seeds from the *Th. turcicum* amphidiploid germinated with 93% producing viable plants.

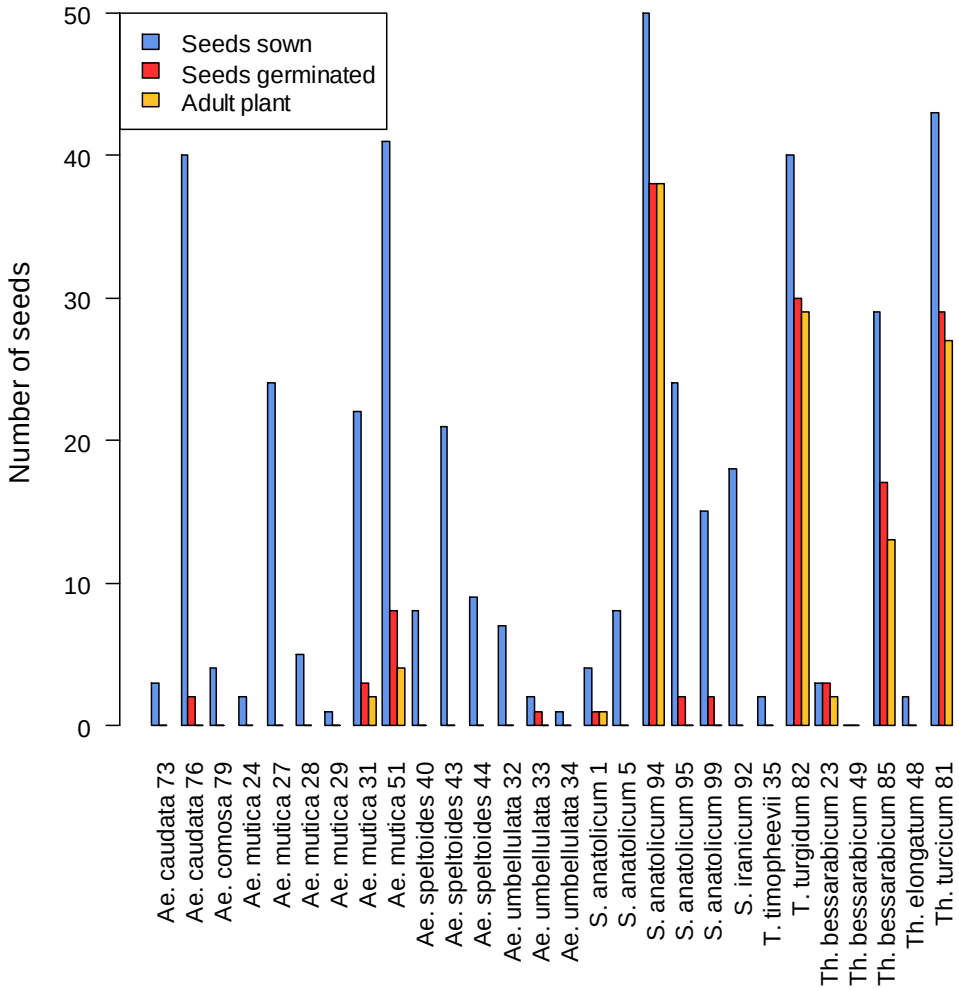


Figure III-9 Number of seeds sown, germinated and number of viable plants

Table III-5 Percentage of successful germination and viable F_1 plant

Amphidiploid	% of F_1 seeds germinated	% of viable F_1 plants
Ae. caudata 76	5% (2/40)	0% (0/2)
Ae. mutica 31	14% (3/22)	67% (2/3)
Ae. mutica 51	20% (8/41)	50% (4/8)
Ae. umbellulata 33	50% (1/2)	0% (0/1)
S. anatolicum 1	25% (1/4)	100% (1/1)
S. anatolicum 94	76% (38/50)	100% (38/38)
S. anatolicum 95	8% (2/24)	0% (0/2)
S. anatolicum 99	13% (2/15)	0% (0/2)
T. turgidum 82	75% (30/40)	97% (29/30)
Th. bessarabicum 23	100% (3/3)	67% (2/3)
Th. bessarabicum 85	59% (17/29)	76% (13/17)
Th. turcicum 81	67% (29/43)	93% (27/29)

III.3.4. Fertility of the F_1 generation

A total of 116 F_1 plants were screened for fertility. Two phenotypes were observed. Some spikes had florets widely open revealing the fluffy stigma and no anthers were observed (**Figure III-10 A**). These spikes did not produce any selfed-seeds and were considered as male sterile. Other spikes presented extruded anthers filled with pollen (**Figure III-10 B**). If at least one selfed-seed was observed on the spike the plant was considered fertile.



Figure III-10 Differences between sterile (A) and fertile (B) flowers and spikes of F_1 resulting from the crosses between CMS and A) *T. turgidum* amphidiploid and B) *S. anatolicum* amphidiploid

Only some F_1s resulting from the crosses between CMS and *S. anatolicum* amphidiploid 94/1 and *Th. turcicum* amphidiploid 81/1/22 were fertile, 16 F_1 and 13 F_1 respectively (**Table III-6**).

Table III-6 Number of fertile F_1 plants for each amphidiploid

Amphidiploid	Total number of F_1 plants	Number of F_1 fertile
Ae. mutica 31	2	0
Ae. mutica 51	4	0
S. anatolicum 1	1	0
S. anatolicum 94	38	16
T. turgidum 82/1	29	0
Th. bessarabicum 23	2	0
Th. bessarabicum 85	13	0
Th. turcicum 81	27	13

Table III-7 presents the fertility results of the F_1 plants resulting from the cross with *S. anatolicum* amphidiploid 94/1. The F_1 plants either resulted from a cross with CMS Spring1 or CMS Spring2. The minimum fertility was 0.03 and 0.01 seeds/spikelet respectively and the maximum was 0.33 and 0.23 respectively. The control (common wheat) set 2.1 seeds/spikelet.

Table III-7 Fertility level of the F_1 plants resulting from the crosses with *S. anatolicum* amphidiploid 94/1 (average of seeds/spikelet per plant)

CMS	No. of fertile F_1 plants	Min. fertility	Max. fertility
Spring1	13	0.01	0.23
Srping2	3	0.03	0.33

Restoration of fertility for the F_1 seed resulting from the cross with the *Th. turgidum* amphidiploid are shown in **Table III-8**. The F_1 plants either resulted from a cross with CMS Winter1 or CMS Winter2. Only one plant with the Winter2 background set seeds with an average of 0.93 seeds/spikelet. In the case of the Winter1 background, the minimum fertility was 0.05 and the maximum 1.29 seeds per spikelet.

Table III-8 Fertility level of the F_1 plants resulting from the crosses with *Th. turcicum* amphidiploid 81/22/8 (average of seeds/spikelet per plant)

CMS	No. of fertile F_1 plants	Min. fertility	Max. fertility
Winter1	12	0.05	1.29
Winter2	1	0.93	0.93

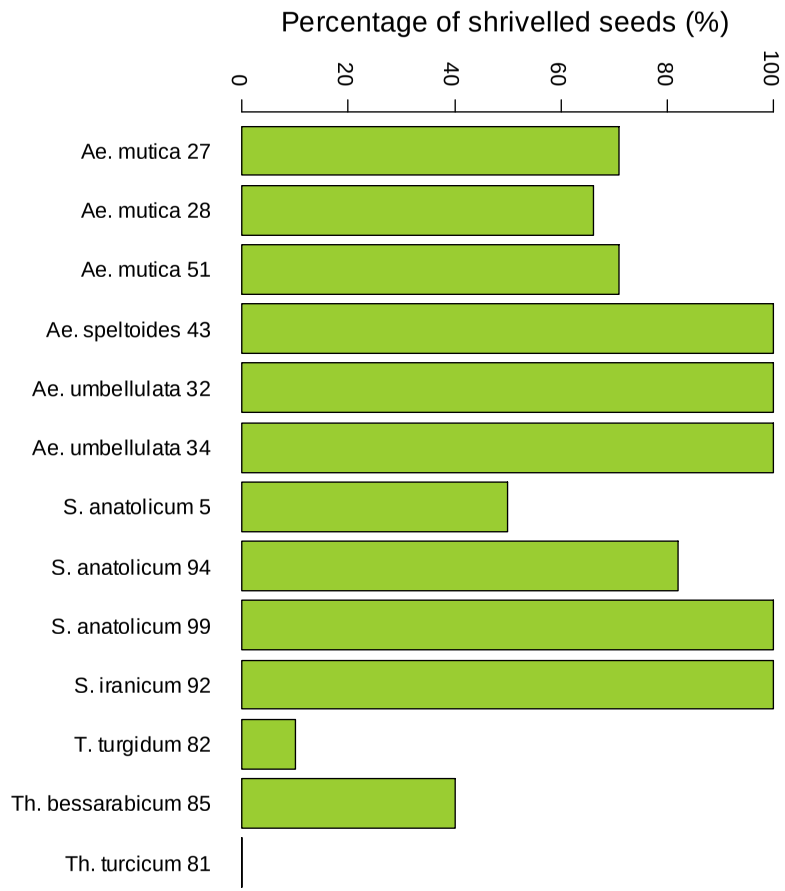
III.3.5. Controls

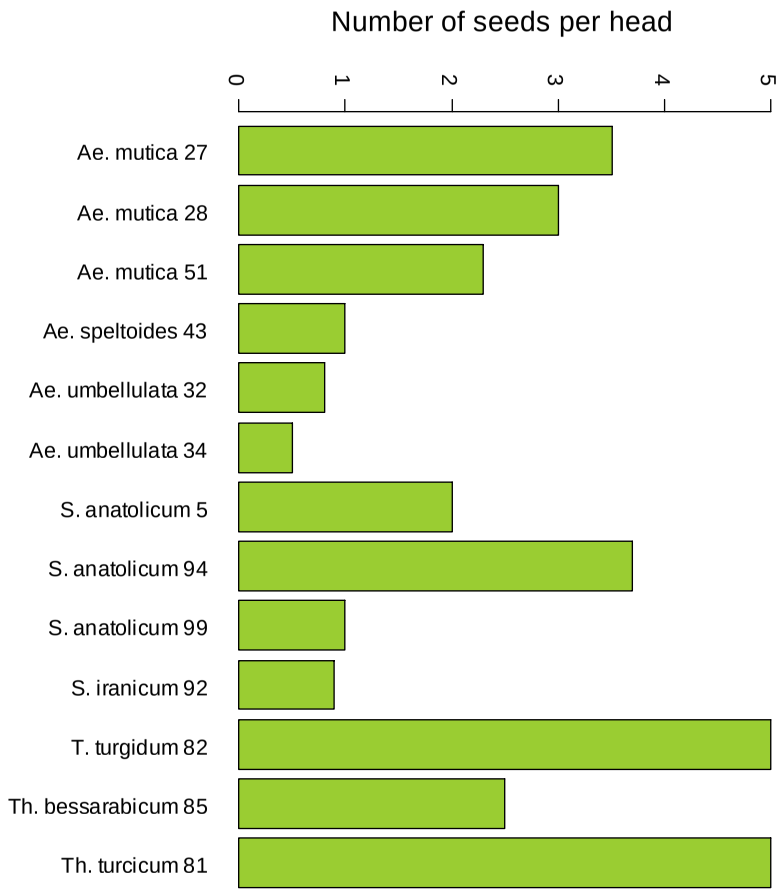
CMS self-fertilised

All heads of the CMS lines which were self-fertilised were sterile.

Common wheat x Amphidiploid

Common wheat genotypes (Chinese Spring, Highbury, Paragon and Pavon 76) were pollinated by the different amphidiploids. The seed set per crosses, the percentage of shrivelled F_1 seeds and the percentage of F_1 seeds that germinated are shown in **Figure III-11**. The results show that only a few combinations led to F_1 seeds. F_1 plants having the following amphidiploids as male parent reached the adult stage: *Ae. mutica* amphidiploids 27/1/21 and 51/1/3, *S. anatolicum* amphidiploid 94/1, *T. turgidum* amphidiploid 82/1, *Th. bessarabicum* amphidiploid 85/1/4 and *Th. turcicum* amphidiploid 81/1/22. All of them were completely sterile.


Percentage of shrivelled seeds (%)
0
20
40
60
80
100
Ae. mutica 27
Ae. mutica 28
Ae. mutica 51
Ae. speltoides 43
Ae. umbellulata 32
Ae. umbellulata 34
S. anatolicum 5
S. anatolicum 94
S. anatolicum 99
S. iranicum 92
T. turgidum 82
Th. bessarabicum 85
Th. turcicum 81


Number of seeds per head
0
1
2
3
4
5
Ae. mutica 27
Ae. mutica 28
Ae. mutica 51
Ae. speltoides 43
Ae. umbellulata 32
Ae. umbellulata 34
S. anatolicum 5
S. anatolicum 94
S. anatolicum 99
S. iranicum 92
T. turgidum 82
Th. bessarabicum 85
Th. turcicum 81

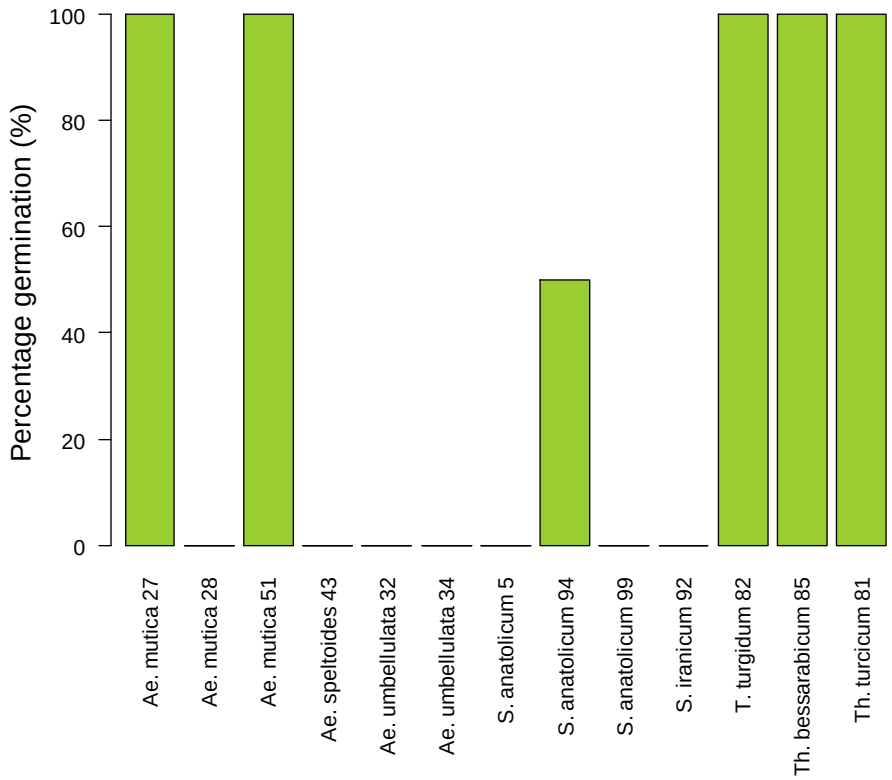


Figure III-11 Results of the crosses between common wheat and amphidiploids A) Seed set, B) Percentage of shrivelled F_1 seeds C) Percentage of germination of the F_1 seeds

CMS x Common wheat

The results of the number of seeds obtained for one head of a CMS line crossed with a common wheat genotype used as the male parent (Chinese Spring, Chinese Spring mutant, Highbury, Paragon and Pavon 76) were shown in **Figure III-12**. An average of 10.8 seeds were set per head when the female was Spring1 variety, 8.0 seeds/head for Spring2, 2.7 for Winter1 and 9.8 for Winter2.

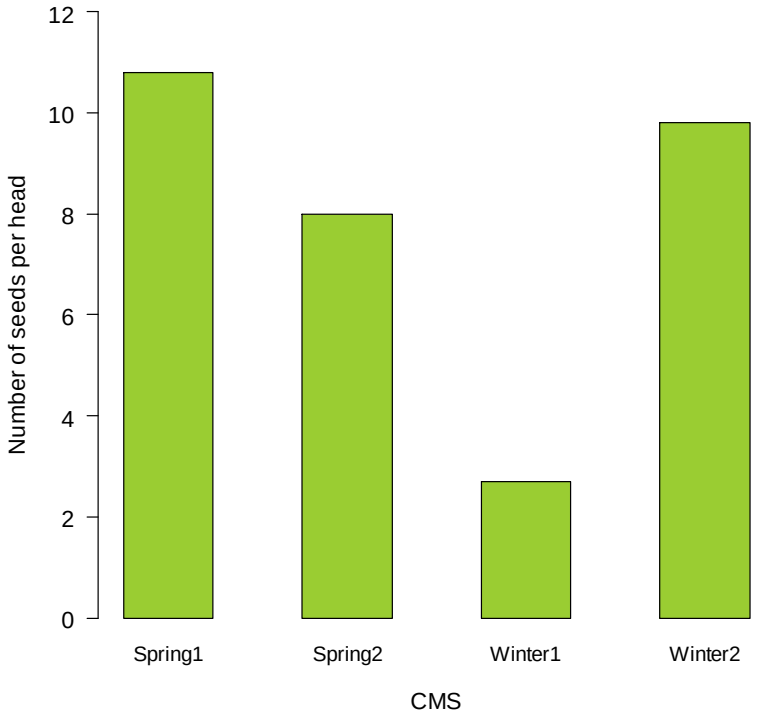


Figure III-12 Number of F_1 seeds per head by crossing CMS line with common wheat pollen

The F_1 seeds had a shape similar to the parental seeds, i.e. none of them where shrivelled. The 34 F_1 were sown and all of them germinated. However, out of all the combinations tested only three of them led to fertile F_1s (**Table III-9**) and all were with Chinese Spring, i.e. on average, the F_1s between CMS Winter1 and Chinese Spring, CMS Winter2 and Chinese Spring, CMS Winter2 and Chinese Spring mutant set 1.23, 0.24 and 0.27 seeds/spikelet respectively.

Table III-9 Average number of seeds per spikelet for the F_1 plants resulting from the crosses between CMS and common wheat

CMS	CS	CS mutant	Highbury	Paragon	Pavon 76
Spring1	0	0	0	0	0
Spring2	0	0	0	0	0
Winter1	1.23	0	0	0	0
Winter2	0.24	0.27	0	0	0

III.4. Discussion

The aim of this chapter was to study wheat interspecific diversity as a potential reservoir of restorer genes against *T. timopheevii* cytoplasm. Wheat/wild relative amphidiploids were chosen as a way to test the whole genome of the wild species. Currently one of the main barriers to the commercial CMS based hybrid wheat is the availability of a total, stable and monogenic restoration against *T. timopheevii* cytoplasm (Sage, 1972). To date, the most common *Rf* genes used are located on the wheat genome but only a combination of 2 or 3 of them has led to a total restoration (Bahl and Maan, 1973; Johnson and Patterson, 1977; Sage, 1976).

The first step consisted of the production of the F_1 hybrids between the CMS line and the wheat/wild relative amphidiploids. The importance of the genetic background on the level of restoration has been shown previously (Maan et al., 1984; Virmani and Edwards, 1983; Wilson and Driscoll, 1983). For that purpose four different commercial varieties from Limagrain were selected as CMS lines: two spring varieties and two winter varieties. Thirty different amphidiploids were used as pollinators, combining thirteen different wild relatives with different spring wheat cultivars (Chinese Spring, Highbury, Paragon and Pavon 76). The wild relative species were selected to cover the interspecific diversity of common wheat including species from *Aegilops*, *Secale*, *Thinopyrum* and *Triticum* genera. Chinese Spring cultivar, a Chinese landrace, has been used worldwide as a genetic and genomic reference basis for wheat from the production of aneuploid stock lines to the genome reference (Liu et al., 2018). Highbury was released in the UK in 1978 and was used for its high yield potential and good bread making qualities (Belderok et al., 2000). Pavon 76, a semi-dwarf variety (*Rht1*) was released for its high grain quality in 1979 by CIMMYT (Waines and Ehdaie, 2007). Finally, Paragon was released by Plant Breeding International, Cambridge and considered as an elite variety in the UK (Angus et al., 2011).

F_1 seeds production

Only 20.7% of the CMS head crossed produced seeds (average of 0.8 seeds per head). Variation of the seed set was observed between the different parental combinations used. For example no F_1 seeds were produced with *S. vavilovii* and one of the *Ae. speltoides* amphidiploids. For other amphidiploids involving wild species such as *Ae. comosa*, *S. iranicum* or *T. timopheevii*, less than 1 seed per head was obtained. However, pollination with some *Ae. caudata*, *Ae. mutica* and *S. anatolicum* amphidiploids led to more than 1 seed per head. *T. turgidum* and *Th. turcicum* amphidiploids led to the best seed set with an average of 4.0 seeds/head. By comparison 7.8 seeds/head were obtained when the CMS line was pollinated with common wheat and 2.4 seeds per head when common wheat (with *T. aestivum* cytoplasm) was pollinated by the pollen of the amphidiploids. The use of wheat/wild relative amphidiploid pollen caused a dramatic decrease of the seed set. However, the number of seeds set per head was not the only criterion to assess the success of the crosses. Seed shape, germination rate and F_1 viability were taken into consideration as well. Out of the 428 F_1 seeds sown, 133 F_1 seeds germinated but only 116 F_1 plants reached the heading stage. For example, despite a seed set of 3.6 seeds/head for *Ae. caudata* amphidiploid 76, 92% of the F_1 seeds were shrivelled, only 5% of the seeds sown germinated and of these, two F_1 plants died in the early development stage. Germination of F_1 involving *Ae. comosa*, *Ae speltoides*, *Ae. umbellulata*, *S iranicum*, *T. timopheevii* and *Th. elongatum* amphidiploids was unsuccessful as well. F_1 seeds which were no-shrivelled were germinated with greater success and mostly reached the adult stage. For example, 75% of the F_1 seeds having *T. turgidum* amphidiploid as pollen parent germinated and 97% of them reached the heading stage. Out of the thirty amphidiploids used as male parent, only eight led to adult F_1s involving the following wild species: *Ae. mutica*, *S. anatolicum*, *T. turgidum*, *Th. bessarabicum* and *Th. turcicum*.

The low seed set, the high percentage of shrivelled F_1 seeds, the low level of germination, the plant lethality and the hybrid sterility are probably the result

of postzygotic barriers leading to hybrid incompatibility (Johnson, 2010). Firstly, one hypothesis underlying the cross sterility is abnormal endosperm development leading to embryo abortion. The two parents used had different ploidy levels. The female parent was hexaploid wheat (AABBDD) and the male parent had various different ploidy levels depending on the genome of the wild species. For example, the *Ae. speltoides* amphidiploid was octoploid (AABBDDSS) while the *T. timopheevii* amphidiploid was decaploid (AABBDDA^{t}A^{t}GG). This difference of ploidy level in the F_1 caused an unbalanced ratio with an excess of the paternal genome. This has also been shown to cause proliferation of the endosperm (Scott et al., 1998; Sekine et al., 2013). It has been demonstrated that an unbalanced genome between the two parents led to the production of shrivelled seeds when the ploidy level of the female line was lower than the ploidy level of the male line (Thompson, 1930). This notion of the direction of the cross has been well documented by Sharma, 1995. In this paper differences in seed set in reciprocal crosses between wheat and wild relatives belonging to *Aegilops, Secale* or *Hordeum* genera were presented. In some cases, interspecific crosses were more successful when species with the lower chromosome number was used as the female line (Sharma, 1995).

An unbalanced genome in the F_1 has been enhanced by the genomic instability of the amphidiploids used as pollen donor. Indeed both chromosome loss and aneuploidy have been detected in both the wheat and wild relative genomes of the amphidiploids. Aberrations occurred mostly in the B genome (Nemeth et al., 2015). The unbalanced genomic composition of the amphidiploids was a consequence of the colchicine treatment which disturbed the microtubule polymerization. An increase of aneuploidy through the generations has also been observed in the case of a *T. aestivum*/*Ae. caudata* amphidiploid (Bluethner et al., 1988).

Germination rates may also have been affected by the presence of gametocidal genes in the genomes of some of the wild relatives. It has been shown that gametocidal chromosomes were present in the *Ae. speltoides* and

Ae. caudata genomes, on chromosome 2S and 3C respectively (Endo, 1990; Tsujimoto and Noda, 1988; Tsujimoto and Tsunewaki, 1984a). Gametes lacking this chromosome were non-viable and the *Gc1a* allele of *Ae. speltoides* also caused endosperm aberration (Endo, 1988; Tsujimoto and Tsunewaki, 1988).

Fertility restoration

Out of the thirteen wild species selected, only five wild species were tested for their ability to restore *T. timopheevii* cytoplasm because of the lack of germination: *Ae. mutica*, *S. anatolicum*, *T. turgidum*, *Th. bessarabicum* and *Th. turcicum*. Total male sterility of the CMS lines has been checked by self-fertilising one head per plant.

The *Th. turcicum* amphidiploid was able to restore *T. timopheevii* cytoplasm in a winter wheat background (fertility level ranging from 0.05 to 1.29 seeds/spikelet). This amphidiploid was the result of the pollination of Chinese Spring by *Th. turcicum* (2n = 10x = 70, EEEEEEEEEE). No restorer gene carried by *Th. turcicum* has previously been reported. However, crosses between the winter CMS line and CS led to fertile F_1 (up to 1.23 seeds/spikelet). Monosomic analysis led to the conclusion that chromosome 1B of CS was carrying a dominant restoring gene causing partial fertility restoration (Kučera, 1982). Chromosome 1B of CS has also been shown to be able to restore the fertility against *Ae. umbellulata* (Tsunewaki, 1974), *Ae. ovata* (syn. *Ae. geniculata*) (Tsunewaki, 1982), *Ae. kotschyi* (Mukai and Tsunewaki, 1979), *Ae. uniaristata* (Mukai, 1983) and *Ae. mutica* (Tsujimoto and Tsunewaki, 1984b) cytoplasm. However, Ahmed et al. (2001) reported that *T. timopheevii* alloplasmic lines with the CS nuclear genome were male sterile. These different conclusions on the ability of CS to restore *T. timopheevii* cytoplasm can be explained by the diversity existing within CS germplasm, as it was thought to be a landrace (Liu et al., 2018). Moreover, the female genotype has been shown to be an important factor for the fertility restoration level (Maan et al., 1984; Virmani and Edwards, 1983; Wilson and Driscoll, 1983). In this study, it was shown that CS was only able to restore winter type CMS lines. Complementary information

to this was that the *Th. turcicum* amphidiploid was revealed to contain only a very few chromosomes of *Th. turcicum* (personal communication Dr. Julie King). The phenotype of this amphidiploid was very similar to CS. Thus the *Th. turcicum* amphidiploid could be considered almost as CS and the presence of a small number of *Th. turcicum* chromosomes caused less genomic conflict in the F_1 generation. However, CS was also used as the female parent in the production of the *S. anatolicum* amphidiploid 1/6/2, *Th. bessarabicum* amphidiploids 23/1/26 and 85/1/85. None of the sixteen F_1 plants involving these three amphidiploids were fertile. However, this sterility could be affected by the genomic instability of the F_1, the loss of the restorer factor or repressing interaction between the different genomes.

Some F_1 plants having *S. anatolicum* amphidiploid 94/1 as the male parent were fertile (fertility level ranging from 0.01 to 0.33 seeds/spikelet). This amphidiploid was the result of the pollination of Pavon76 by *S. anatolicum* (2n = 2x = 14, RR). As the F_1s between the CMS and Pavon 76 were male sterile, fertility restoration observed in the F_1 generation must have been caused by *Rf* factors carried by the *S. anatolicum* genome. This was supported by the work of Curtis and Lukaszewski, 1993, who mapped two restorer factors on the rye genome: *Rfc3* on chromosome 6RL and *Rfc4* on chromosome 4RL. However, out of the 38 F_1 from *S. anatolicum* amphidiploid 94/1 that germinated, only 16 plants were fertile. In addition one F_1 having *S. anatolicum* amphidiploid 1/6/2 as the male parent reached the adult stage but did not produced any F_2 seeds. The sterility of the other 21 F_1 plants could be the result of the loss of the chromosome carrying the restorer factor or genomic instability leading to hybrid sterility.

Conclusion

Wheat/wild relative amphidiploids have been used as a bridge to facilitate the crosses between common wheat and its wild species, allowing a quick, broad and easy test of the wild genome. However, this did not remove post zygotic barriers preventing the formation of a new polyploid: unbalanced and unstable

genomes, deleterious genes. Only five out of the thirteen different species selected were screened for their ability to restore *T. timopheevii* cytoplasm. *S. anatolicum* was the only one found to carry *Rf* genes. Hybrid sterility of the F_1 hybrids involving other wild species could hide the presence of other *Rf* genes. The next step will be to study in more detail the restoration factor(s) carried by *S. anatolicum*. Discovery of new restorer factors in the wild relative genomes could also be achieved by using a panel of introgression lines covering the complete genome of the wild relative as pollinator for the CMS lines, in order to remove the post-zygotic barriers. Moreover, some chromosomes of the wild species were not present in the amphidiploids, chromosomes which were potentially carriers of *Rf* genes.

IV. Chapter 4: Study of the restoration of *T. timopheevii* cytoplasm by *S. anatolicum*

Summary

The aim of this chapter was to study the ability of *S. anatolicum* to restore fertility of plants with *T. timopheevii* cytoplasm. F_1 plants produced in Chapter 3 (CMS lines x wheat/*S. anatolicum* amphidiploid), were selfed several times to stabilize and individualize *S. anatolicum* chromosomes and isolate the restorer chromosome(s). Characterization of the *Rf* chromosome was done by cytogenetics methods (GISH and FISH) combined to genotyping (KASP markers). We showed that long arm of chromosome 4R of *S. anatolicum* carries a *Rf* gene causing partial restoration up to 1.75 seeds/spikelet. However unstable expression of the fertility was observed. In addition, highest fertility level was observed in the presence of additional chromosomes implying that modifier loci might be present on *S. anatolicum* genome.

IV.1. Introduction

IV.1.1. Taxonomy of the genus *Secale*

The genus *Secale* is a small but economically important genus. It includes annuals and perennials, outbreeders and inbreeders, wild, feral and cultivated species which originated from Turkey, Transcaucasia, Iran and central Asia (Hagenblad et al., 2016). The species are mainly diploid (2n = 4x = 14, RR) but some tetraploid forms exist (Frederiksen and Petersen, 1998; Lupton, 1987). The taxonomy and phylogeny of the genus *Secale* have been the subject of many discussions leading to different classifications. Based on morphology and geographical information, the genus was firstly separated into 14 species (Roshevitz, 1947; Vavilov, 1926). Then, the use of cytogenetic methods led to a four species classification: *S. montanum*, *S. cereale*, *S. sylvestre* and *S. vavilovii* (Hammer et al., 1987; Khush, 1962; Vences et al., 1987). The most recent classification is based on only three species*: S. sylvestre*, *S. montanum* (*S. strictum*) and *S. cereale*, in which *S. vavilovii* has been included (**Table IV-1**)

(Frederiksen and Petersen, 1998; Sencer and Hawkes, 1980). This classification has been confirmed by analysis of the genetic diversity using SNPs (Hagenblad et al., 2016), SSRs (Maraci et al., 2018; Ren et al., 2011) and GBS (Schreiber et al., 2018).

S. sylvestre is a wild annual autogamous species, very strongly isolated from the other species as it differs in its morphology, vegetative structure and low crossability with the other *Secale* species (Khush, 1962; Maraci et al., 2018). The wild perennial outcrossing *S. strictum* includes the subspecies *S. africanum*, *S. anatolicum*, *S. chaldicum*, *S. ciliatoglume* and *S. kuprijanovii*. The *S. cereale* group includes non-shattering weeds (*S. cereale*), semi-shattering weeds (*S. afghanicum*, *S. dighoricum* and *S. segetale*) and completely shattering weeds (*S. ancestrale*, *S. vavilovii* and *S. iranicum*) (Kilian et al., 2009).

Table IV-1 Classification of the genus *Secale*

Species	**Subspecies**	**Comment**
S. sylvestre	*S. sylvestre*	Annual, autogamous
S. strictum = *S. montanum*	*S. montanum* *S. anatolicum* *S. ciliatoglume* *S. kuprijanovii* *S. africanum*	Perennial, outbreeder (except *S. africanum*)
S. cereale	*S. cereale* *S. vavilovii* *S. ancestrale* *S. segetale* *S. afghanicum* *S. dighoricum* *S. iranicum*	Annual, outbreeder

Many meiotic studies of the hybrids between the different species highlighted the presence of several translocations between the members of the genus *Secale.* The *S. cereale* genome differs by two large reciprocal translocations

from the genome of *S. montanum* (Khush, 1962; Khush and Stebbins, 1961; Krüger, 1947; Riley, 1955; Stutz, 1957) and a third small translocation might be present (Khush and Stebbins, 1961; Singh and Röbbelen, 1977). *S. montanum* and *S. sylvestre* genomes differ by a small translocation (Khush, 1962). *S. montanum* and *S. vavilovii* differ by a large translocation (Khush and Stebbins, 1961; Singh and Röbbelen, 1977). *S. vavilovii* shares with *S. cereale* the same translocation which distinguishes them from *S. strictum*, confirmation of the three species classification (Al-Beyroutiová et al., 2016; Cuadrado and Jouve, 2002; Frederiksen and Petersen, 1998). Three chromosomes have been identified as being involved in the translocations which differentiate the genome of *S. montanum* from *S. cereale* and *S. vavilovii*: the 2R, 6R and 7R chromosomes (Heemert and Sybenga, 1972; Singh and Röbbelen, 1977; Sybenga et al., 1985). These results have been confirmed by the description of the different chromosomal rearrangements during the *Triticeae* genome evolution (Devos et al., 1993). The translocation between 2R and 6R was confirmed by localisation of the *Sec2* locus on chromosome 2R of rye and chromosome $6R^{m}$ of *S. montanum* (Shewry et al., 1985) and $6R^{arf}$ of *S. africanum* (Li et al., 2015a).

These conclusions allowed the history of the genus *Secale* to be drawn up. Many studies agreed to consider *S. sylvestre* as the oldest species of the genus from which the other species have evolved (Chikmawati et al., 2005; De Bustos and Jouve, 2002; Hammer et al., 1987), while another hypothesis considered *S. cereale* as the hybrid of *S. montanum* and *S. sylvestre* (Stutz, 1957) but this has been rejected. More recently, *S. montanum* has been considered as the oldest species. *S. sylvestre* is thought to have differentiated from *S. montanum* during the Miocene while the differentiation of *S. cereale* occurred at a much earlier geological time, during the Pleistocene, and *S. africanum* migrated to South Africa (**Figure IV-1**) (Cuadrado and Jouve, 2002; Khush, 1962). *S. cereale* originated from *S. montanum* by the progressive fixation of the two translocations (Khush and Stebbins, 1961).

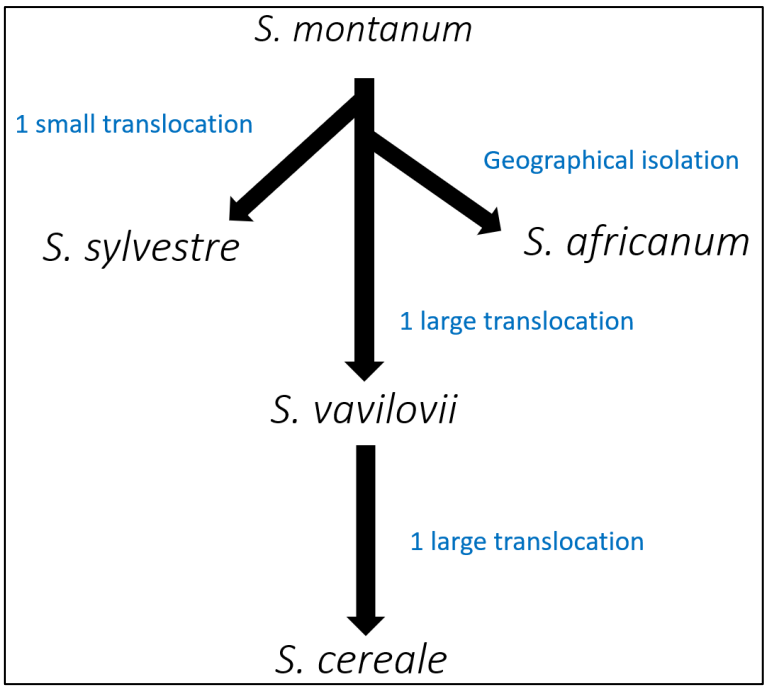


Figure IV-1 Probable derivation of the different *Secale* genomes from a common ancestral genome identical to that of *S. montanum*

IV.1.2. Aim and objectives for the chapter

The aim of this chapter was to explore in more depth the results of the previous chapter, i.e. the ability of the *S. anatolicum* genome to restore the fertility against *T. timopheevii* cytoplasm:

- To individualize the restorer factor(s) carried by *S. anatolicum*
- To identify the restorer(s) chromosomes using genomic and genotyping methods.

IV.2. Material and Methods

IV.2.1. Plant material

F_1 lines resulting from the crosses between spring CMS common wheat line (with *T. timopheevii* cytoplasm) and wheat/*S. anatolicum* amphidiploids produced in Chapter 3 were used as the initial material.

For the genomic *in situ* hybridization and genotyping analysis different Chinese Spring addition lines were ordered from the Germplasm Resource Unit at JIC: CS/*S. cereale* var. Imperial (1R to 7R) (Driscoll, 1971), CS/*S. cereale* var. King (1R to 7R) (Miller, 1973) and CS/*S. montanum.* Only CS/*S. montanum* addition

lines for chromosomes $1R^m$, $2R^m$, $4R^m$, $5R^m$ and $6R^m$ have been established (Miller, 1973).

IV.2.2. Crossing program

The crossing scheme is illustrated in **Figure IV-2**. At least one head of each plant was selfed and in case of any sign of fertility restoration, one head was backcrossed with wheat pollen (refer to Chapter II.1.4). The selfed and back-crossed seeds coming from the fertile plants were germinated and screened for fertility restoration. This process was carried out until the F_4 and BC_1F_2 generation.

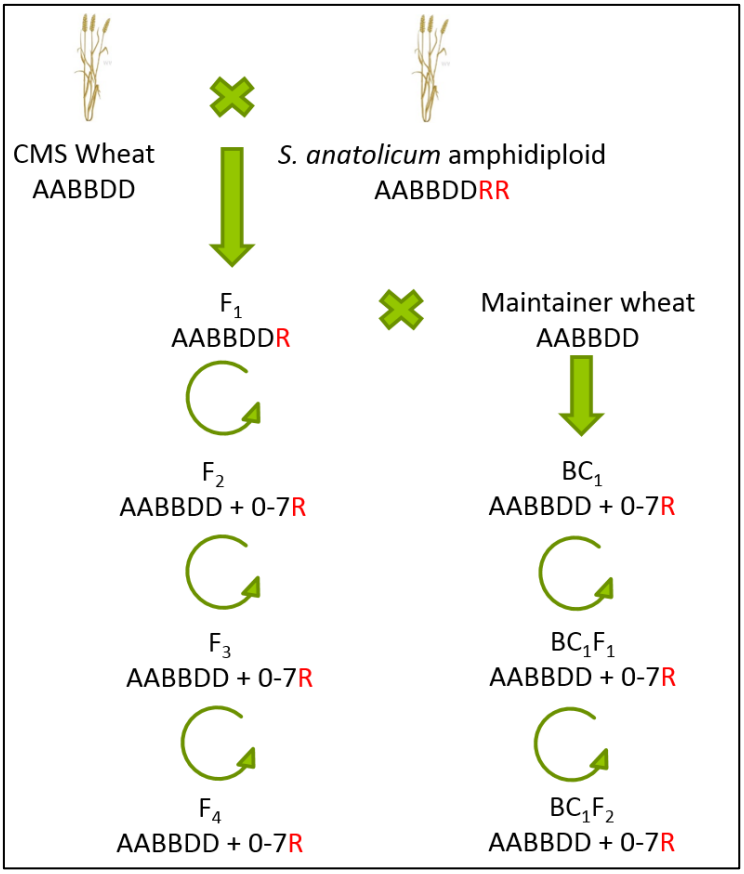


Figure IV-2 Crossing scheme to individualize *S. anatolicum* restorer chromosomes against *T. timopheevii* cytoplasm

IV.2.3. Seed germination

Germination of the seeds was carried out according to the protocol (refer to Chapter II.1.1). Once the seed germinated, it was sown into soil (John Innes

compost No. 2). As the initial crosses were done with CMS spring wheat, the plants were put into vernalisation for four weeks (at 8°C with 12 hours light per day). Plants were grown in the glasshouse (at 21°C, 16 hours light per day).

IV.2.4. Assessment of the fertility restoration

To score male fertility restoration, the heads of the F_1s were bagged prior to anthesis and harvested after ripening. The seed set of one head was defined as the number of seeds divided by the number of spikelets. Seed set of one plant was the average of the seed set of the selfed spikes. A plant without any selfed seed was considered as male sterile, whereas a plant with at least one seed was considered as male fertile.

IV.2.5. Cytogenetic analysis

Preparation of metaphase spreads was described in Chapter II.3.3. Slides were probed as follows. Single colour GISH was carried out using total genomic DNA of common wheat as blocking DNA and total genomic DNA of *S. anatolicum* labelled by nick translation with Alexa Fluor 546-14-dUTP (Invitrogen; C11401) (gold) as probe. For the multicolour GISH, total genomic DNA of *Ae. speltoides* was used as blocking DNA and total genomic DNA of *S. anatolicum* labelled by nick translation with Alexa Fluor 546-14-dUTP (Invitrogen; C11401) (gold), total genomic DNA of *T. urartu* labelled by nick translation with Alexa Fluor 488-5-dUTP (Invitrogen; C11397) (green) and total genomic DNA of *Ae. tauschii* labelled with Alexa Fluor 594-5-dUTP (Invitrogen; C11400) (red) were used as probes. Multicolour FISH was done using the two repetitive DNA sequences pSc119.2 (McIntyre et al., 1990) and pAs.1 (Bedbrook et al., 1980). They were labelled with Alexa Fluor 488-5-dUTP (green) and Alexa Fluor 594-5-dUTP (red) respectively. All slides were counterstained with DAPI.

IV.2.6. KASP markers genotyping

A total of 1000 KASP markers, polymorphic between the four wheat cultivars and the ten wild relatives (one of which is *S. cereale*), have been developed by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) in

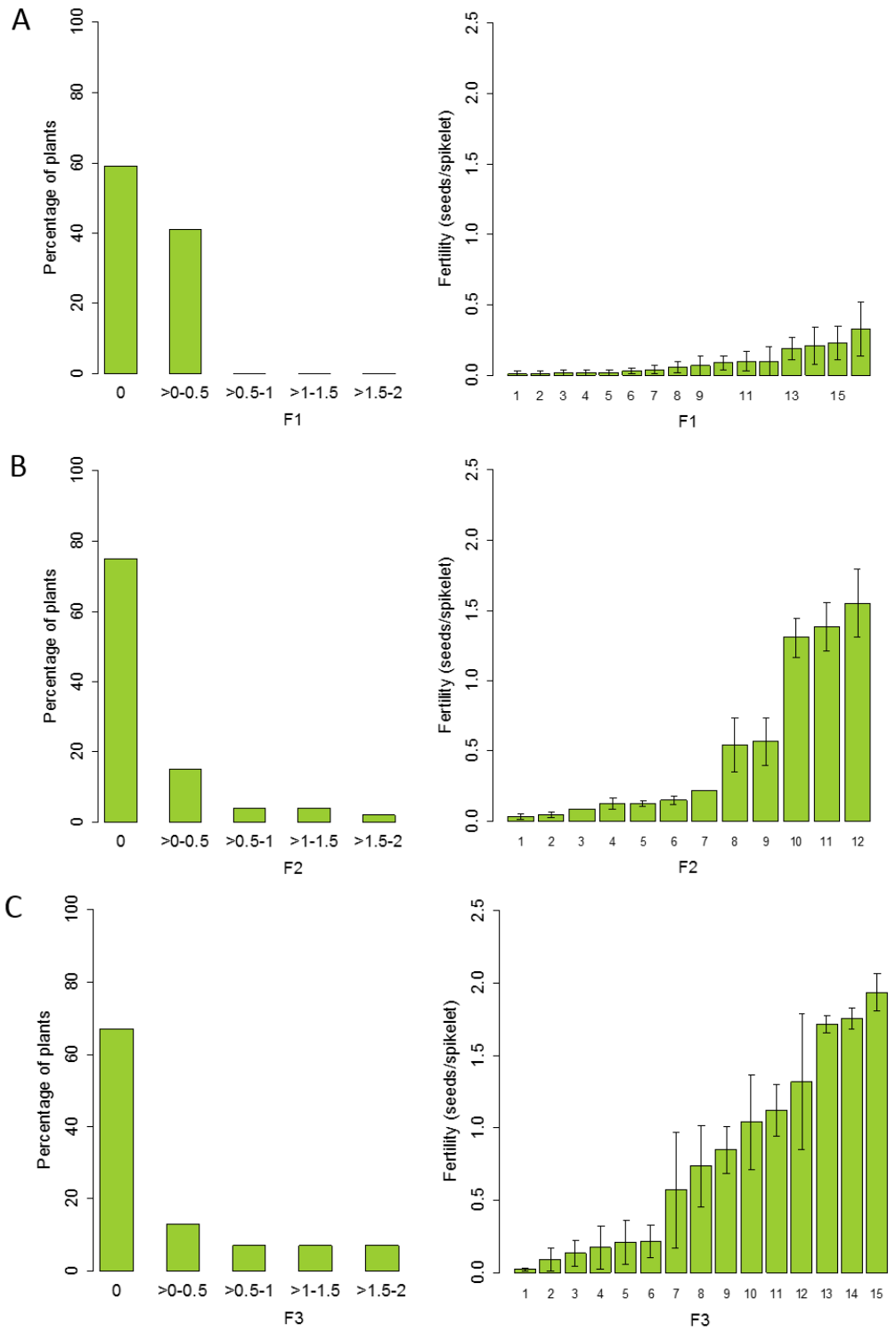
collaboration with LGC. These 1000 KASP markers have been validated on 5000 genotypes, including the wild accession of *S. anatolicum* and the wheat parents. Out of the markers validated, those polymorphic between the different wheats and the wild *S. anatolicum* accession were selected leading to a set of 198 markers. 142 of the markers were assigned to a rye linkage group according to previous genotyping carried out by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham). From these 142 markers, a total of 103 markers were selected to cover the seven linkage groups of the *Secale* genome. Genotyping was carried on the CS/rye addition lines (1R to 7R), CS/*S. montanum* addition lines ($1R^m$, $4R^m$, $5R^m$ and $6R^m$) and the newly developed fertile lines carrying *S. anatolicum* chromosome(s) (based on GISH results). Genomic DNA extraction was done by the method explained in Chapter II.2. PCRs were run by the protocol explained in Chapter II.4.2. Each mixture was composed of 5 µL of DNA, 2.43 µL of dH_2O, 2.50 µL of ROX PACE mix and 0.068 µL of primers mix. The details of the PCR cycle were given in Chapter II.4.2.

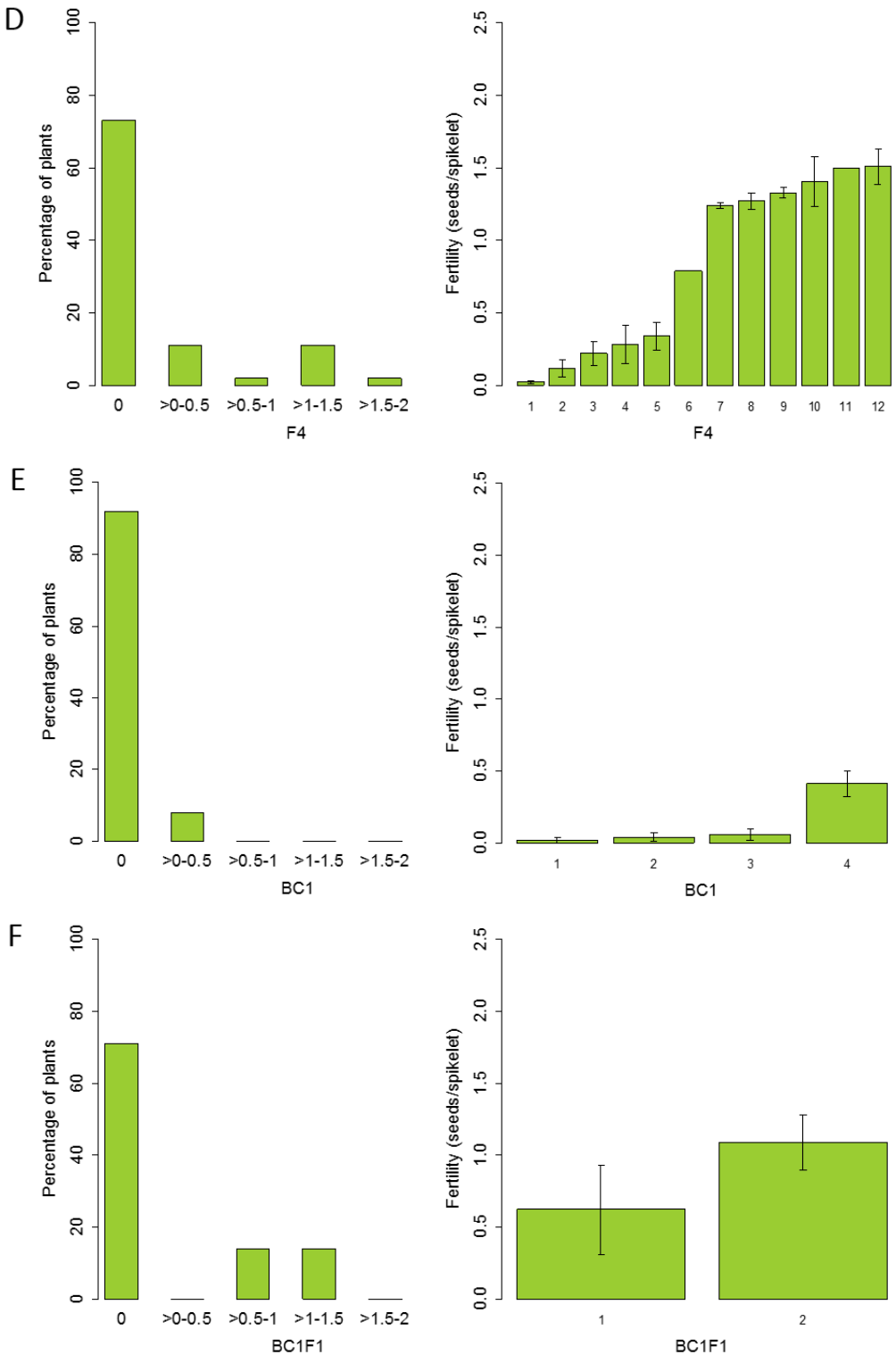
IV.3. Results

IV.3.1. Scoring of the fertility

The fertility results for the F_1, F_2, F_3, F_4, BC_1, BC_1F_1 and BC_1F_2 generations are shown in **Figure IV-3**. Within the F_1 generation, 41% (16 plants) were fertile, but setting less than 0.5 seed/spikelet with a maximum of 0.33 seeds (**Figure IV-3 A**). 25% of the F_2 plants (12 plants) were fertile with a fertility ranging between 0.03 and 1.56 seeds/spikelet. The majority of them (75%) set less than 1 seed/spikelet (**Figure IV-3 B**). 33% of the F_3 plants (15 plants) were fertile with a fertility ranging from 0.02 to 1.94 seeds/spikelet (**Figure IV-3 C**). 27% of the F_4 plants (12 plants) were fertile, 50% of them set more than 1 seed/spikelet with a maximum of 1.51 (**Figure IV-3 D**). Only 4 BC_1 plants were fertile (8%), setting a maximum of 0.41 seeds/spikelet (**Figure IV-3 E**). 28% of the BC_1F_1 plants (two plants) were fertile, with 0.62 and 1.19 seeds/spikelet (**Figure IV-3 F**). Within the BC_1F_2 generation 12% of the plants were fertile (4 plants) with a fertility ranging from 0.31 to 1.42 seeds/spikelet (**Figure IV-3 G**).

Spring wheat, used as control, set 2.3 seeds/spikelet. Important variation of the fertility level within the same individual are reflected by the error bars. For example, for the F_3 individual number 7 (**Figure IV-3 C**) two heads were sterile and one head set 1.71 seeds/spikelet.




D
Percentage of plants
0
20
40
60
80
100
0
>0-0.5
>0.5-1
>1-1.5
>1.5-2
F4
Fertility (seeds/spikelet)
0.0
0.5
1.0
1.5
2.0
2.5
1
2
3
4
5
6
7
8
9
10
11
12
F4
E
Percentage of plants
0
20
40
60
80
100
0
>0-0.5
>0.5-1
>1-1.5
>1.5-2
BC1
Fertility (seeds/spikelet)
0.0
0.5
1.0
1.5
2.0
2.5
1
2
3
4
BC1
F
Percentage of plants
0
20
40
60
80
100
0
>0-0.5
>0.5-1
>1-1.5
>1.5-2
BC1F1
Fertility (seeds/spikelet)
0.0
0.5
1.0
1.5
2.0
2.5
1
2
BC1F1

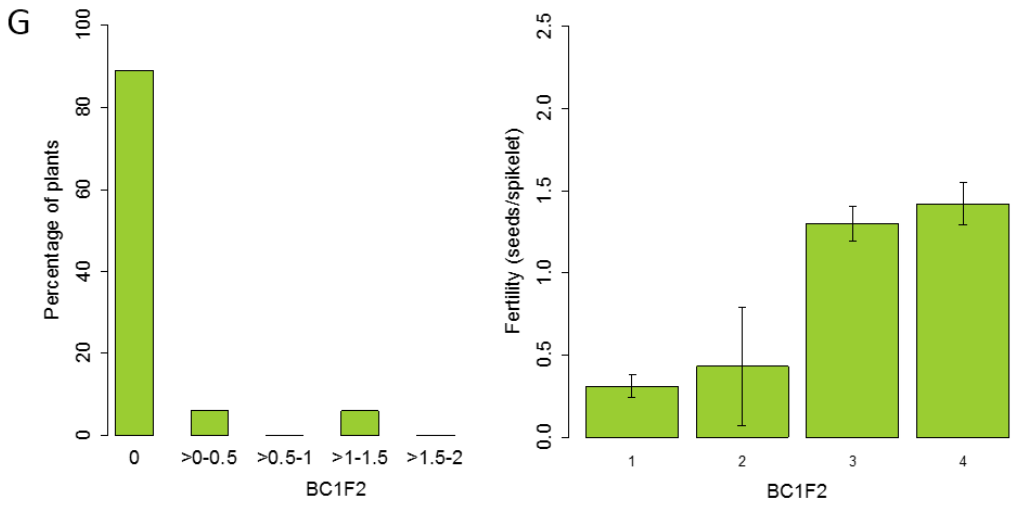


Figure IV-3 Distribution of the fertility (left hand side graphic) and level of fertility scored (right hand side graphic) for the generations: A) F_1, B) F_2, C) F_3, D) F_4, E) BC_1, F) BC_1F_1, G) BC_1F_2

IV.3.2. Cytogenetic analysis

GISH analysis was carried out on the *S. anatolicum* amphidiploid 94/1 and on the F_3, BC_1F_1, F_4 and BC_1F_2 generations in order to determine their genomic composition.

IV.3.2.1. *Genomic composition of the S. anatolicum amphidiploid*

Multicolour GISH was carried out on metaphase cells of the wheat/*S. anatolicum* amphidiploid used as the original male parent (**Figure IV-4**). By analysing several lines, the genomic constitution was established as 14 A-genome chromosomes, 14 B-genome chromosomes, 12 D-genome chromosomes and various numbers of *S. anatolicum* chromosomes ranging from 8 to 14.

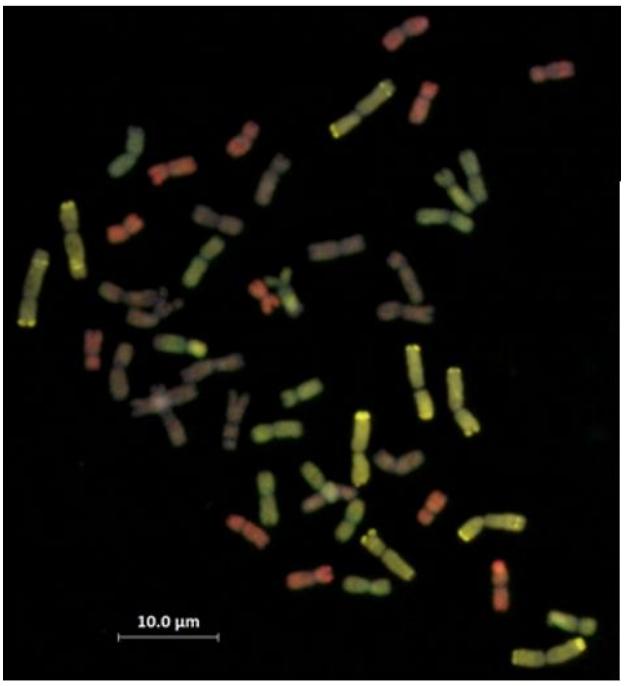


Figure IV-4 Multi-colour GISH image showing the genomic composition of a wheat/*S. anatolicum* amphidiploid carrying *S. anatolicum* chromosomes
A genome in green, B genome in blue, D genome in red and R genome in gold

IV.3.2.2. *S. anatolicum chromosome segregation*

Chromosomes of *S. anatolicum* segregated through the crossing scheme (**Table IV-2**). 35 F_3 plants carried a range of the *S. anatolicum* genome. No translocation lines were detected, only addition or substitution lines with up to 4 complete *S. anatolicum* chromosomes. Moreover, 19 of these plants also carried telocentric *S. anatolicum* chromosomes. One F_3 plant carried a single *S. anatolicum* chromosome. Seven BC_1F_1 plants grew in the glasshouse and 3 of them carried a single complete chromosome of *S. anatolicum*. 26 of the 44 F_4 plants carried a range of *S. anatolicum* chromosomes up to a maximum of a telocentric plus 6 complete *S. anatolicum* chromosomes present in one line. Eighteen of these plants carried a single telocentric *S. anatolicum* chromosome. In the BC_1F_2 plants, 11 out of the 35 germinated had a fraction of the *S. anatolicum* genome. Seven plants carried a single complete chromosome of *S. anatolicum*. Three spontaneous translocation lines were detected, one in the F_4 generation and 2 in the BC_1F_2 generation. In all cases,

half of a chromosome of *S. anatolicum* had been translocated with either the B-genome or D-genome of wheat (**Figure IV-5**).

Table IV-2 Segregation of the *S. anatolicum* chromosomes through the generations

	F_3	**BC_1F_1**	**F_4**	**BC_1F_2**
Total number of plants	45	7	44	35
No. of plants with *S. anatolicum* chromosome(s)	35	3	26	11
Minimum number of *S. anatolicum* chromosomes	0.5	1	0.5	0.5
Maximum number of *S. anatolicum* chromosomes	4	1	6	2
No. of plants with a telocentric *S. anatolicum* chromosome	19	0	17	1
No. of plants with a single *S. anatolicum* chromosome	1	3	0	7
No. of plants with a translocation	0	0	1	2

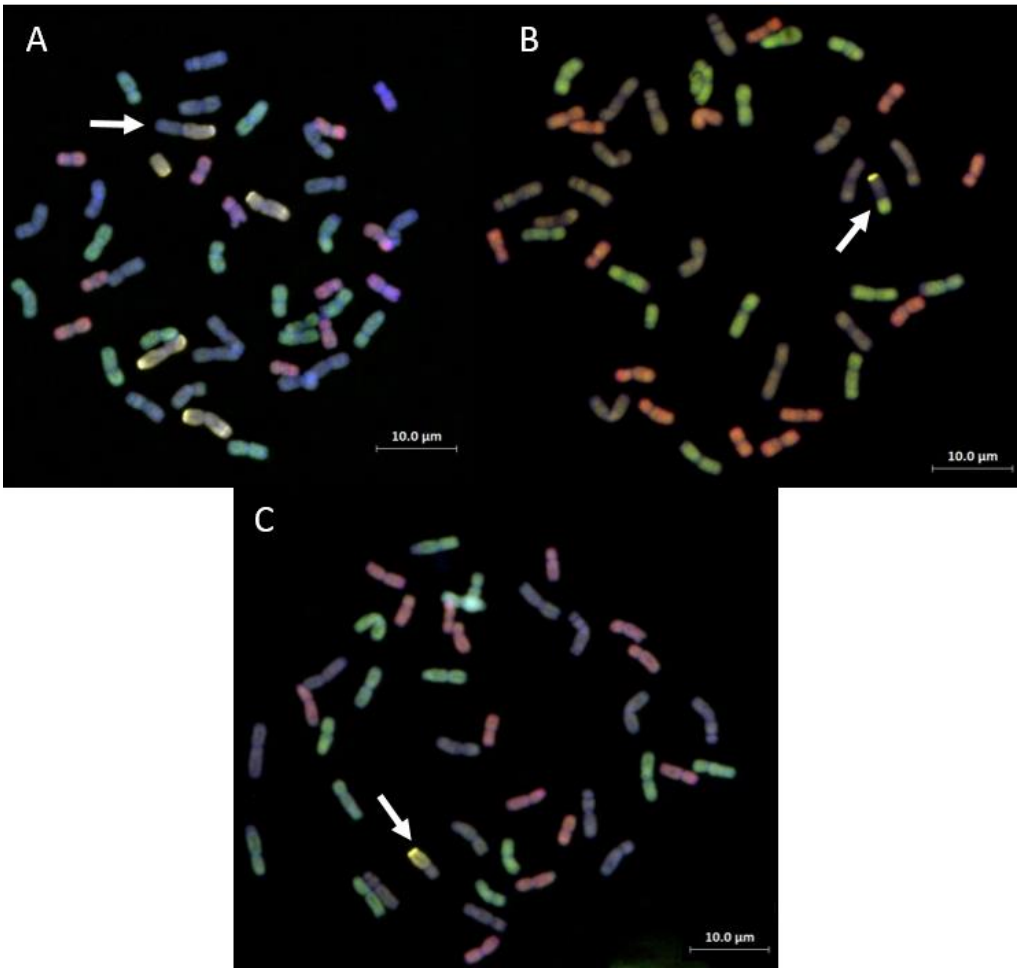


Figure IV-5 Multi-colour GISH images showing intergenomic translocations between *S. anatolicum* and the B and D genomes of wheat

A) F_4, B) and C) BC_1F_2. A genome in green, B genome in blue, D genome in red and R genome in gold. The intergenomic translocations are indicated by the white arrows

IV.3.2.3. *Wheat genome chromosome number*

The number of wheat chromosomes, i.e. A, B and D genomes, varied between the individuals in the different generations. The A and B genomes had their complete chromosome set (14) whereas between 12 and 14 D-genome chromosomes were present. For example, the metaphase spread of the F_4 plant shown in **Figure IV-5 A** shows a genome constitution of 14 A-genome chromosomes (green), 14 B-genome chromosomes (blue) and 12 D-genome chromosomes (red).

IV.3.3. Transmission of the *S. anatolicum* chromosomes

Table IV-3 and **Table IV-4** presents the percentage transmission of *S. anatolicum* chromosomes from generation F_3 to F_4 and BC_1F_1 to BC_1F_2 respectively. When a single *S. anatolicum* chromosome was present in the F_3 plant, 92% of the offspring lost it and the rest carried only half of this chromosome. When two chromosomes of *S. anatolicum* were present in the F_3 generation, both were kept in only 25% of the selfed-offspring. The remaining 75% plants carried between 0.5 and 1.5 *S. anatolicum* chromosomes. Three complete *S. anatolicum* chromosomes were transmitted to the next generation in only 25% of cases. The other seeds exhibited higher chromosome numbers. Offspring resulting from the selfing of plants with 4 *S. anatolicum* chromosomes lost all these chromosomes in 28% of the case, had less than 4 for 48% of them, kept the 4 chromosomes for 12% of them and in 12% of the offspring a higher chromosome number was observed. In the case of the BC_1F_1, when a single *S. anatolicum* chromosome was present. 69% of the BC_1F_2 plants lost this chromosome, 8% had half of it, 20% carried it complete and 1 plant was homozygous for this chromosome.

Table IV-3 Transmission of *S. anatolicum* chromosome from F_3 to F_4

No. of *S. anatolicum* chromosomes in F_3	**1**	**2**	**2.5**	**3**	**4**
No *S. anatolicum* chromosome left in F_4	92% (11/12)				28% (7/25)
Lower *S. anatolicum* chromosome number in F_4 compared to F_3	8% (1/12)	75% (3/4)	100% (1/1)	25% (1/4)	48% (12/25)
Same *S. anatolicum* chromosome number in F_4 compared to F_3		25% (1/4)			12% (3/25)
Higher *S. anatolicum* chromosome number in F_4 compared to F_3				75% (3/4)	12% (3/25)

Table IV-4 Transmission of *S. anatolicum* chromosome from BC_1F_1 to BC_1F_2

	BC_1F_1 plants with a single *S. anatolicum* chromosome
No *S. anatolicum* chromosome left in BC_1F_2	69% (24/35)
Telocentric *S. anatolicum* chromosome in BC_1F_2	8% (3/35)
1 *S. anatolicum* chromosome number in BC_1F_2	20% (7/35)
2 *S. anatolicum* chromosomes number in BC_1F_2	2% (1/35)

IV.3.4. Combination of fertility scoring and GISH results

All plants which did not carry any chromosomes of *S. anatolicum* were completely sterile. The most fertile F_3 line set 1.94 seeds/spikelet and carried one complete chromosome and two telocentric chromosomes of *S. anatolicum* (**Figure IV-6 A**). The most fertile F_4 plant set 1.51 seeds/spikelet and had one complete chromosome and a telocentric chromosome of *S. anatolicum* (**Figure IV-6 B**). The BC_1F_1 line which showed the highest seed set of 1.09 seeds/spikelet carried one complete chromosome of *S. anatolicum* (**Figure IV-6 C**). The most fertile BC_1F_2 line set 1.42 seeds/spikelet and carried 2 complete homozygous chromosomes of S. *anatolicum* (**Figure IV-6 D**).

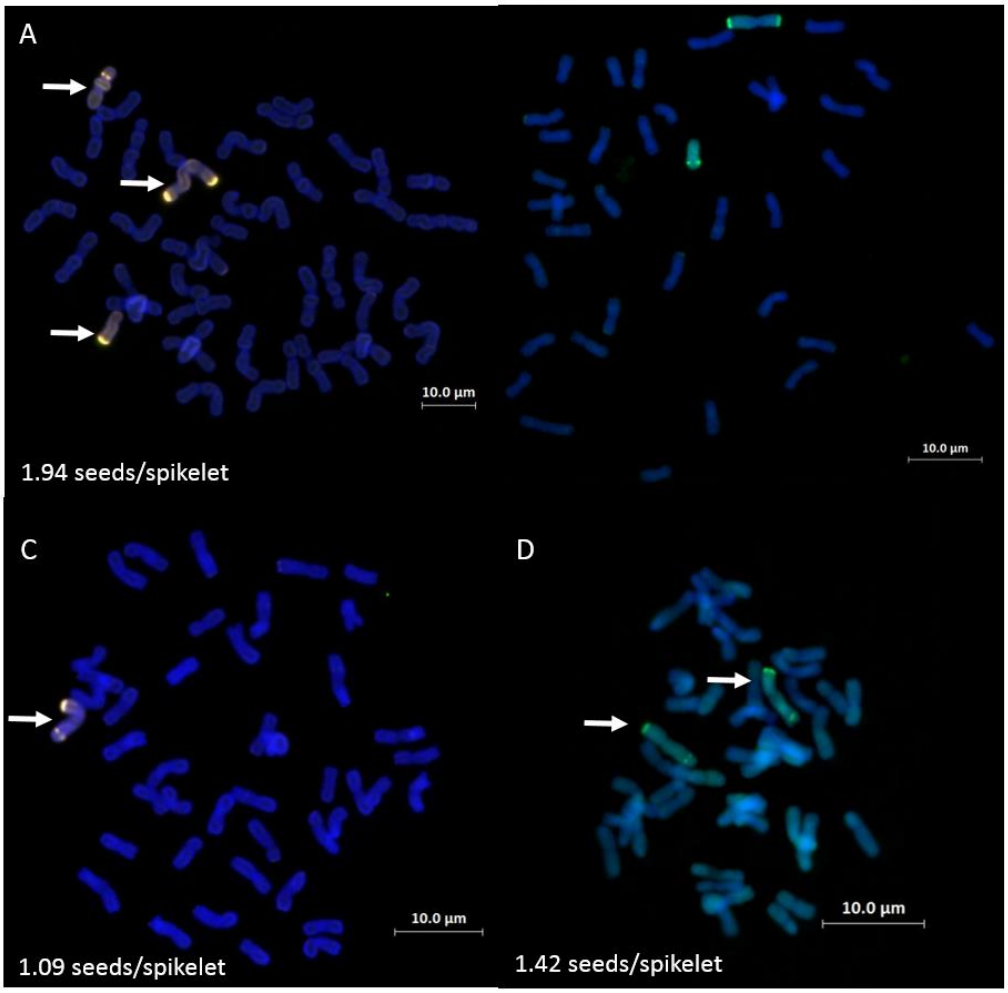


Figure IV-6 Single colour GISH image showing metaphases of the lines with the highest fertility with labelled genomic DNA of *S. anatolicum*

The wheat genome is coloured in blue and *S. anatolicum* genome in green or gold (indicated by the white arrows). A) F_3 genotype, 1.94 seeds/spikelet. B) F_4 genotype, 1.51 seeds/spikelet. C) BC_1F_1 genotype, 1.09 seeds/spikelet. D) BC_1F_2 genotype, 1.42 seeds/spikelet

A total of eleven plants with a single chromosome of *S. anatolicum* were produced (**A** and **Table IV-5**). Out of them, six were fertile with a fertility ranging from 0.31 to 1.75 seeds/spikelet. Five plants carried only a telocentric chromosome of *S. anatolicum* (**B**). Two of them, in the F_4 generation, were fertile with an average of 0.03 and 1.27 seeds/spikelet.

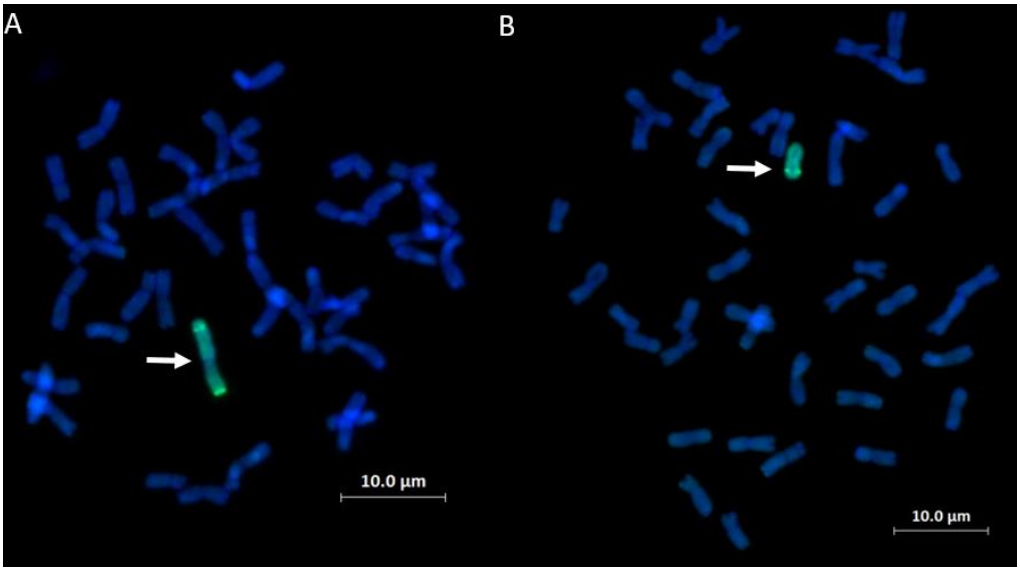


Figure IV-7 Single colour GISH image showing metaphase of fertile lines with A) one complete and B) telocentric chromosome of *S. anatolicum*
The wheat genome is coloured in blue, the *S. anatolicum* genome in green (white arrows)

Table IV-5 Fertility level for the plants in relation with the number of chromosomes of *S. anatolicum*

		F_3	BC_1F_1	F_4	BC_1F_2
1 complete *S. anatolicum* chromosome	Total no. of plants	1	3	0	7
	No. of fertile plants	1	2	0	3
	Lowest fertility	1.75	0.62		0.31
	Highest fertility	1.75	1.09		1.3
Telocentric *S. anatolicum* chromosome	Total no. of plants	0	0	4	1
	No. of fertile plants			2	0
	Lowest fertility			0.03	
	Highest fertility			1.27	

IV.3.5. Characterisation of the *S. anatolicum* chromosome carrier of the fertility factor by *in situ* hybridization

By analysing the chromosomes in metaphase cells of fertile plants, the systematic presence of one chromosome of *S. anatolicum* with the same pattern was noticed. This chromosome was identified by its pattern, i.e. a large telomeric band on the short arm and two small dots at the lower part of the

long arm (**Figure IV-8**). This chromosome was named as the *Rf* chromosome for the rest of this chapter.

To characterize the *Rf* chromosome, FISH using the probe psc119.2 followed by single colour GISH analysis was carried out on the CS/rye and the available CS/*S. montanum* addition lines. In addition, all the $2R^m$ addition lines analysed were revealed to have lost the *S. montanum* chromosome. Results showed that all chromosomes could be distinguished from each other (**Figure IV-9**). The production of a karyotype from this analysis, will be helpful for the identification of the *Rf* chromosome. For *S. montanum*, chromosomes $1R^m$ and $4R^m$ had signal at the terminal regions of both arms. Probe psc119.2 hybridized at the terminal region of the short arm of chromosome $5R^m$. Chromosome $6R^m$ showed a strong signal at the telomeric extremity of its short arm and two dots on the long arm. In rye signal patterns were observed mostly at the extremity of the short arm of chromosomes 3R, 4R, 5R, 6R and 7R. Several signals were dispersed along chromosomes 1R, 6R and 7R. Chromosomes 3R, 4R and 5R were characterised by two signals on their long arm. Probe psc119.2 did not hybridize with chromosome 2R.

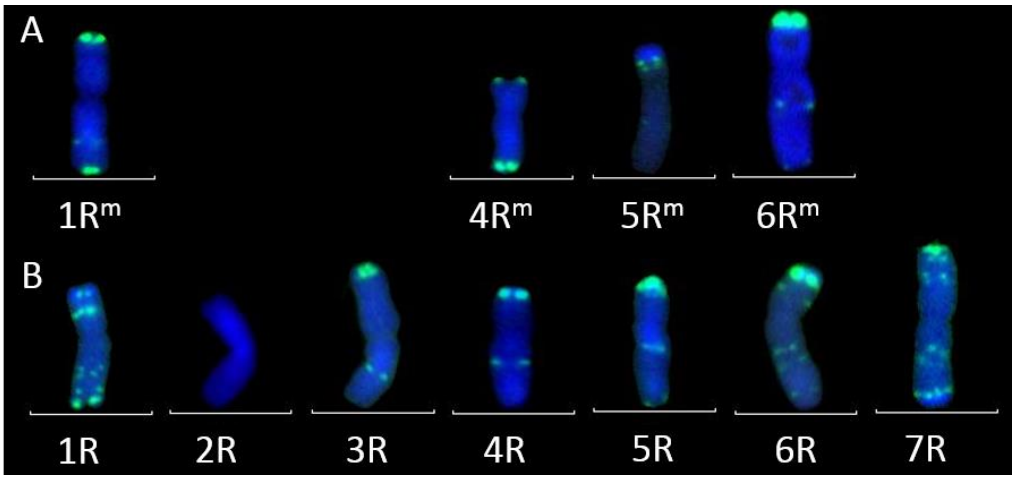


Figure IV-9 Development of a FISH-based karyotype for A) *S. montanum* and B) *S. cereale* by using the probe pSc119.2

FISH followed by GISH analysis was carried out on the fertile lines to identify the *Rf* chromosome (**Figure IV-10**). The pattern of this chromosome was

compared with the karyotype of *S. montanum* and *S. cereale* (**Figure IV-11**). The *Rf* chromosome of *S. anatolicum* showed a very similar pattern to chromosome 4R of *S. cereale* and $6R^m$ of *S. montanum*. Moreover, the *Rf* factor seemed to be located on the long arm of these chromosomes as shown by the presence of a telocentric chromosome in some of the lines (**B**).

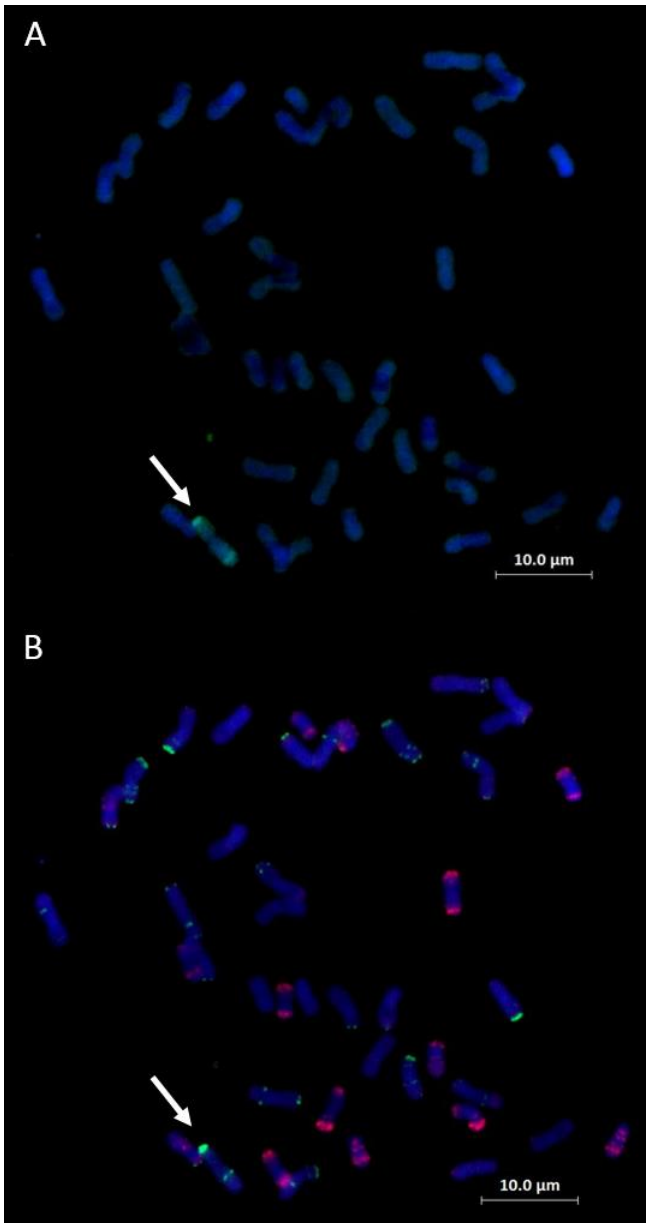


Figure IV-10 Results of GISH and FISH to characterize the *Rf* chromosome
A) Single colour GISH of a fertile line with a single chromosome of *S. anatolicum* (green, indicated by the white arrow). B) Multi-colour FISH of the same line (*S. anatolicum* chromosome indicated by the white arrow).

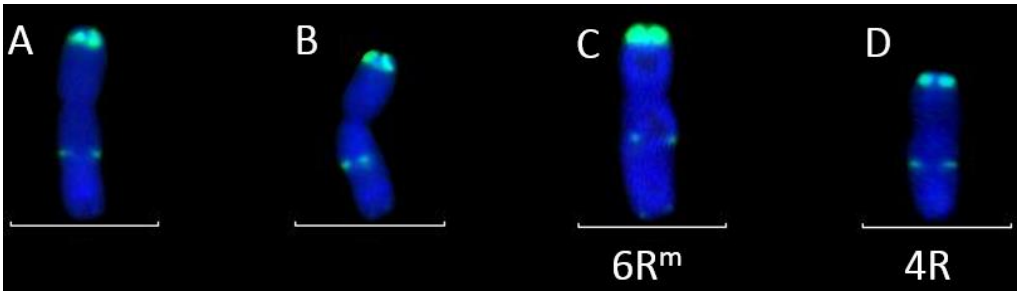


Figure IV-11 FISH analysis using psc119.2 as probe
A) and B) *Rf* chromosome of S*. anatolicum*, C) Chromosome $6R^m$ of *S. montanum* and D) Chromosome 4R of rye

The F_1 resulting from the cross of the CMS and CS/rye addition lines were scored for their fertility. Lines with chromosome 5R were all sterile whereas lines with 4R were fertile. No line with chromosome 6R was obtained.

IV.3.6. Characterisation of the *S. anatolicum* chromosome carrier of the fertility factor by genotyping

KASP markers were available in the summer of 2018. Out of the 103 KASP markers tested, 31 did not amplify correctly or did not show any polymorphism between wheat and the *S. anatolicum* genome (**Table IV-6**). A total of 72 polymorphic markers between wheat and wild *S. anatolicum* were left covering the whole *Secale* genome (Table IV-6).

Table IV-6 Number of KASP markers available, tested and amplified for each *Secale* linkage group

Linkage group	No. of polymorphic markers	No. of markers tested	No. of markers which amplified
1	25	15	9
2	24	17	13
3	18	13	12
4	18	18	9
5	23	13	8
6	24	17	13
7	10	10	7
Total	142	103	72

The position of the markers were confirmed by the genotyping results for the addition lines. For that reason, two markers were repositioned, one from chromosome 2R to 6R and one from chromosome 7R to 4R (**Figure IV-12**). Within a linkage group, some markers were reordered, in order to get a heterozygous block. All the fertile lines produced carry either the complete 4R chromosome (lines 1 to 7, 10, 11 and 13) or the long arm of chromosome 4R (lines 8, 9, 12, 14 and 15). Other chromosomes were present in some lines. Line 7 also carried chromosomes 1R and 2R. The small arm of chromosome 3R was detected in line 8. Line 9 had a complete 2R chromosome. Line 10 was composed of a fragment of the short arm of chromosome 1R and the complete 2R chromosome. The presence of a complete 2R chromosome was detected in the lines 11, 12 and 13.

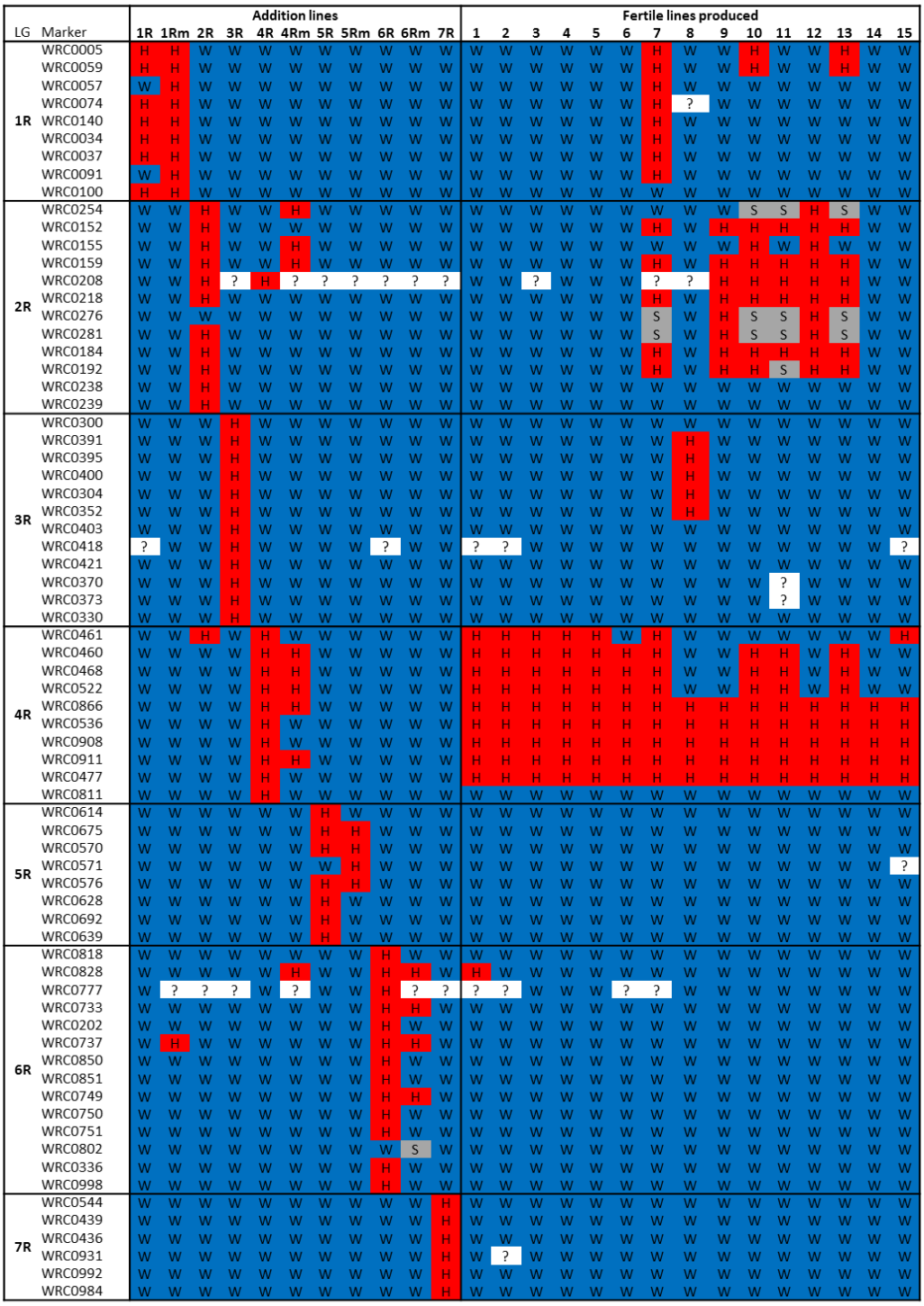


Figure IV-12 Graphical representation of the KASP genotyping of the CS/*Secale* addition lines and the fertile lines

Markers are ordered by linkage groups and are scored as "W" for wheat allele, "H" for heterozygous and "S" for *Secale* allele. Missing values are indicating by "?"

Genotyping results for the CS/*S. montanum* addition lines showed that all the markers of chromosome 1R were located on chromosome $1R^m$. However, the markers from the short arm of chromosome 2R were located on the short arm

of chromosome $4R^m$ (no information is available for chromosome $2R^m$ as this addition line was not available). Not of all of the markers specific to chromosome 4R were polymorphic between *S. montanum* and the wheat genome and only some markers from chromosomes 5R and 6R were located on chromosomes $5R^m$ and chromosome $6R^m$ respectively.

IV.3.7. Comparison between cytogenetics and genotyping results

Table IV-7 summarizes the cytogenetic and genotyping results. The results from the two techniques were consistent.

Table IV-7 Comparison of the number of *S. anatolicum* chromosome detected by GISH and *S. anatolicum* chromosomes characterized by KASP

Line	GISH	KASP
1	1	4R
2	1	4R
3	1	4R
4	1	4R
5	2	4R
6	1	4R
7	4	1R + 2R + 4R
8	1	3RS + 4RL
9	1+2*0.5	2R + 4RL
10	3.5 + 1 translocation	1RS + 2R + 4R
11	3 + 3*0.5	2R + 4R
12	1 + 0.5	2R + 4RL
13	4	1RS + 2R + 4R
14	0.5	4RL
15	0.5	4RL

The **Figure IV-13** presents three examples. In line 2, GISH detected 1 complete chromosome and all the markers heterozygous between wheat and *S. anatolicum* were located on chromosome 4R. GISH revealed the presence of a

telocentric *S. anatolicum* chromosome in line 15 and only the markers of the long arm of chromosome 4R were found to be heterozygous between wheat and *S. anatolicum*. One complete and one telocentric *S. anatolicum* chromosome were detected in line 12 which were the complete 2R chromosome (indicated by the yellow arrow) and the long arm of chromosome 4R (indicated by the white arrow).

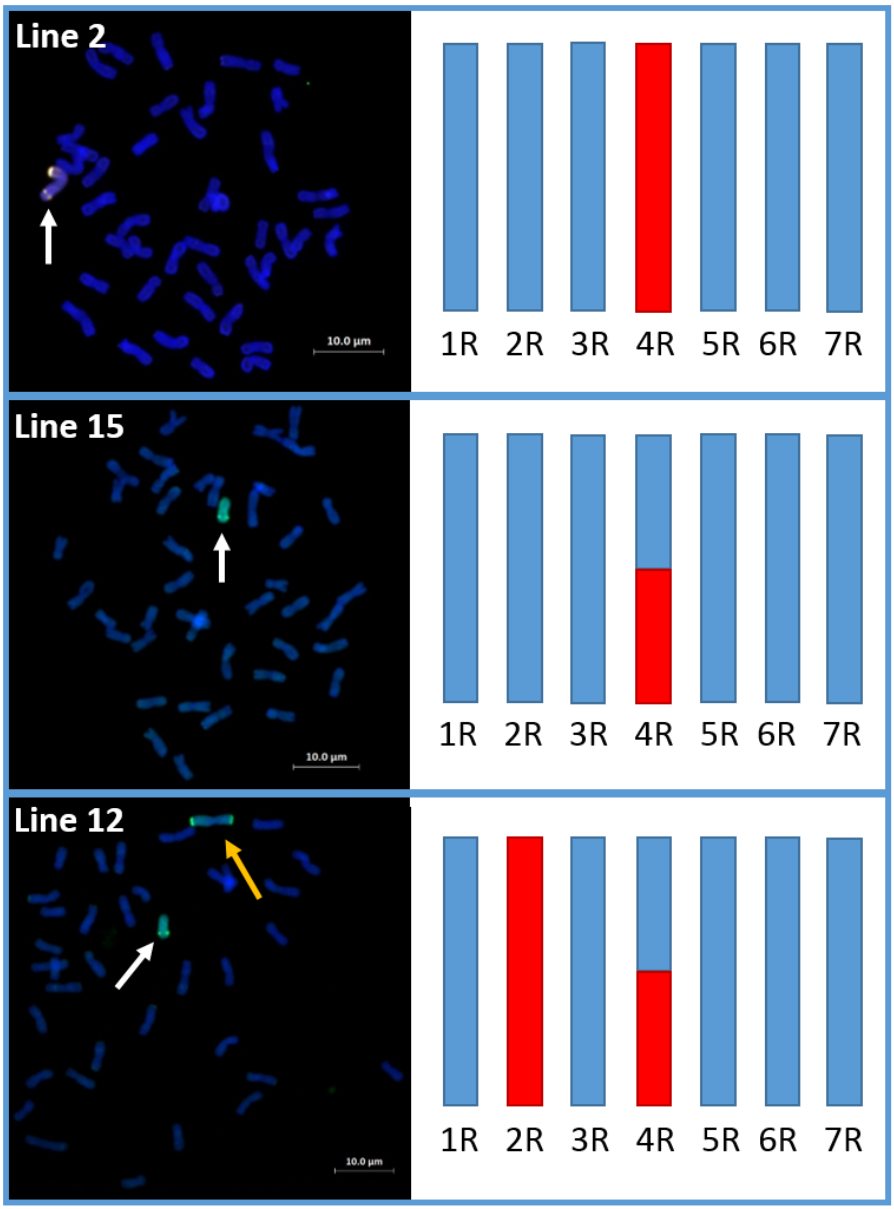


Figure IV-13 Comparison between GISH and KASP marker results of some fertile lines

On the left, single colour GISH image, the wheat genome is coloured in blue and the *S. anatolicum* genome in green or gold (indicated by the arrows). On the right are the genotyping results, a blue colour indicates the wheat allele and a red colour the *S. anatolicum* allele.

The combination of GISH and genotyping data was needed in some cases to conclude about the genomic composition of lines. For example, with KASP markers no distinction was possible between line 4 and 5, both characterized as carriers of chromosome 4R of *S. anatolicum* (**Table IV-7**). However, GISH analysis of line 4 revealed that it carried one copy of 4R while line 5 had two complete 4R chromosomes. Another example was line 8 which had a complete *S. anatolicum* chromosome which appeared to be 4R from cytogenetics analysis. However, KASP markers revealed that this chromosome was in fact composed of the small arm of chromosome 3R and the long arm of chromosome 4R.

IV.3.8. Phenotyping of the lines

Differences in the phenotype of the lines produced were observed **(Figure IV-14**). Independently to the presence of *S. anatolicum* genome in the plant, dwarf plants were noticed. Height measurements gave an average of 39 cm for the F_4 plants and 56 cm for the BC_1F_2 generation (57 cm for normal wheat).

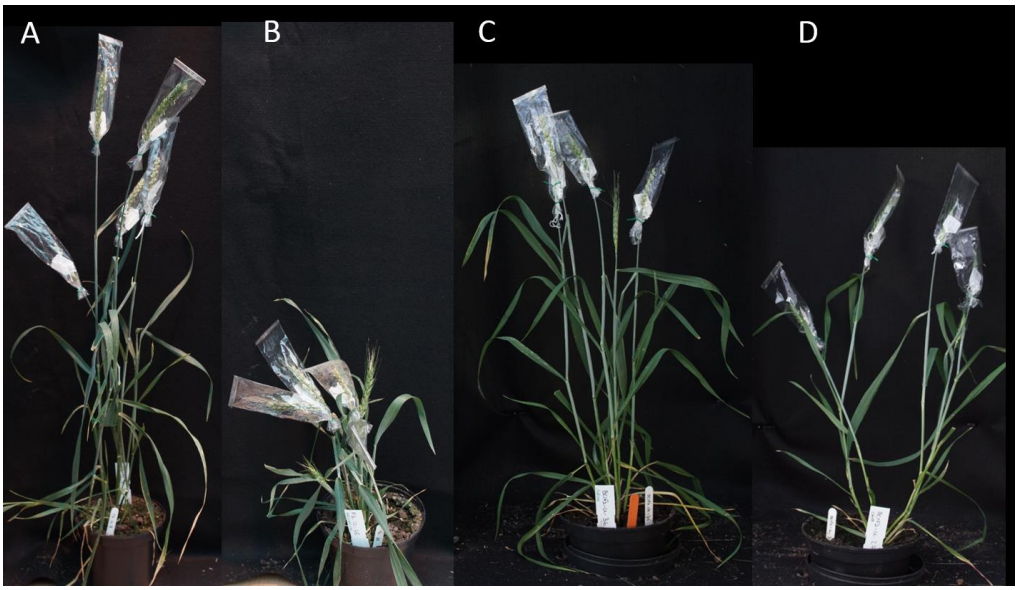


Figure IV-14 Phenotype of some lines produced
A) F_3 line, B) F_4 line, C) and D) BC_1F_2 lines

IV.4. Discussion

The aim of this chapter was to study the ability of *S. anatolicum* (accession *P208/141*) to restore fertility of plants with *T. timopheevii* cytoplasm. F_1 plants produced in Chapter 3 (CMS lines x wheat/*S. anatolicum* amphidiploid), were selfed several times to stabilize and individualize *S. anatolicum* chromosomes and isolate the restorer chromosome(s). This strategy has previously been used successfully to study the effects of individual rye chromosomes on wheat (O'mara, 1940).

Restoration of fertility level

Fertility restoration was observed in successive generations with an increase in the fertility level from the F_1 to the F_3 generation (maximum of 0.33 and 1.94 seeds/spikelet respectively). The fertility of the F_4 generation (1.51 seeds/spikelet) was lower than the F_3 (maximum of 1.94 seeds/spikelet). Fertile F_1 plants were, in parallel, back-crossed with wheat and went through a process of selfing as well. The maximum fertility restoration level increased from 0.41 seeds/spikelet for the BC_1 to 1.42 seeds/spikelet for the BC_1F_2 generation. The decrease of the fertility between the F_3 and the F_4 generations could be due to genome rearrangements causing sterility. It has been shown that chromosomal aberrations occur in the wheat chromosomes of the progeny of wheat/rye hybrids. These alterations included deletions, duplications, translocations and inversions of wheat chromosomes (Fu et al., 2013; Silkova et al., 2017; Tang et al., 2014). Through the selfing generations, these aberrations accumulated in the lines causing higher sterility. For example, in the presence of chromosome 7R, three 4A chromosomes were observed (Fu et al., 2013). Aneuploidy causes important changes in DNA methylation pattern and for that reason is often associated with reduced fitness (Gao et al., 2016; Henry et al., 2010; Zhang et al., 2013). The vegetative phenotype of the F_4 lines could be the consequence of such a genome aberrations with a drastic height reduction with an average of 39 cm compared to a control of 57 cm. Moreover, the instability of the genome was enhanced by the lack of two D-genome chromosomes in the amphidiploid used as the

male parent for the initial cross. These two chromosomes were still missing in the F_4 lines. However, by back crossing the F_1 (instead of selfing), the line recovered a complete D–genome. Production of wheat/*S. anatolicum* translocation lines could also help to stabilize the genome. Three spontaneous translocations were detected, two fragments of the *S. anatolicum* were inserted into the B-genome of wheat and one into the D-genome. The spontaneous translocation of a complete arm was the result of a centric breakage-fusion named as a Robertsonian translocation was also observed. During meiosis in a monosomic substitution line, the two univalents (one rye chromosome and one wheat chromosome) can break at their centromere and then fuse to form a new chromosome (Qi et al., 2007; Sears, 1952). Ninety six percent of the translocations found between rye and wheat chromosomes in a Triticale x wheat population were centric break-fusion types (Lukaszewski and Gustafson, 1983). Unfortunately, in this work these translocations did not involve the restorer chromosome. By screening a larger population, the probability of discovering a translocation involving the *Rf* chromosome would be increased. This strategy will now be possible with the development of the KASP markers since the summer 2018.

Isolation of the *Rf* chromosome

The *S. anatolicum* chromosomes segregated through the successive selfing generations. The F_1 plants were composed of a complete set of wheat chromosomes (42 chromosomes) and a haploid set of *S. anatolicum* chromosomes. During meiosis, the *S. anatolicum* univalents were either lost in the cytoplasm or only distributed to half of the gametes (O'mara, 1940; Silkova et al., 2013). It has been shown previously that the *Secale* genome is less competitive than the wheat genome and thus will be preferentially eliminated during meiosis (Silkova et al., 2011b, 2017). The result was the production of lines carrying a single *S. anatolicum* chromosome. Some of these lines were fertile. The highest fertility restoration level was observed in the F_3 generation with 1.75 seeds/spikelet. As all the lines carrying only wheat chromosomes were sterile, the *S. anatolicum* chromosome isolated was a carrier of one *Rf*

factor. In some cases, lines with a telocentric *S. anatolicum* chromosome were fertile (maximum of 1.27 seeds/spikelet in the F_4 generation).

Characterization of the *Rf* chromosome

Characterization of the *Rf* chromosome was firstly done using cytogenetic techniques (GISH and FISH) (Xu et al., 2016). Ideally, FISH analysis should have been carried out on wheat/*S. anatolicum* addition lines to produce a karyotype allowing the identification of the *Rf* chromosome. Unfortunately, such lines were not available. Addition lines of other species of the genus *Secale* were chosen, i.e. *S. montanum* and *S. cereale*. However, these species differed from each other by different chromosome rearrangements (Khush, 1962). *S. montanum* has been considered as the oldest species of the genus. As it belonged to the same species as *S. anatolicum*, their genomes were considered as very similar. The addition lines of rye were characterized as well. Rye differed from the *S. montanum* species by at least two large translocations involving chromosomes 2R, 6R and 7R (Heemert and Sybenga, 1972; Khush, 1962; Khush and Stebbins, 1961). The probe pSc119.2 was chosen for the karyotyping as it has been demonstrated that it hybridizes to the telomeric region in all the species of the *Secale* genus and thus allows an unambiguous identification (Cuadrado and Jouve, 1997). FISH analysis of the fertile lines led to the conclusion that a chromosome showing the same pattern was always present. Its signal pattern was very similar to chromosome $6R^m$ of *S. montanum* and chromosome 4R of rye. More precisely, the restorer factor seemed to be located on the long arm of this chromosome.

Confirmation of these results was done by molecular analysis. The most fertile lines and the fertile line carrying a single complet telocentric *S. anatolicum* were genotyped using the KASP markers developed by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham). As the markers had been previously validated for the rye genome, both CS/rye and CS/*S. montanum* addition lines were added as controls. A total of 103 KASP markers spread across the seven linkage groups were run. 72 markers amplified correctly,

showing polymorphism between wheat and the *S. anatolicum* genome. Genotyping results of the different addition lines showed an important similarity between the rye and *S. anatolicum* genomes. However, very few markers were found in common with *S. montanum*. While *S. anatolicum* belongs to the *S. montanum* species, important diversity differences among the subspecies have been identified. For example, a yellow rust resistance gene is located on chromosome $6R^m$ of *S. montanum* (Schneider et al., 2016) and 2RL of *S. montanum* ssp. *africanum* (Lei et al., 2013). In addition, SSR analysis of 139 different accessions of the *Secale* genus showed that the *S. montanum* and *S. anatolicum* accessions originating from Iran were closer to the *S. cereale* species than the *S. montanum* and *S. anatolicum* accessions were to each other, suggesting that gene flow between members of the different species occurred (Maraci et al., 2018). It is possible that the accession of *S. anatolicum* used in this chapter was closer to rye than to the *S. montanum* accessions. Moreover, some authors have suggested *S. anatolicum* as the ancestor of *S. cereale* (Lorenz and Kulp, 1991). Genotyping results confirmed the cytogenetics analysis, i.e. the long arm of chromosome 4R of *S. anatolicum* is involved in the fertility restoration. As chromosome 4R is considered as the most conserved among the rye chromosomes (Cuadrado and Jouve, 1995), an analogy can be carried out with chromosome 4R of rye. It has been shown that chromosome 4RL of rye carries a restorer gene against *T. timopheevii* cytoplasm, designated *Rfc4* (Curtis and Lukaszewski, 1993). *Rfc4* partially restored the fertility (up to 1.65 seeds/spikelet) which is similar to the results obtained here with the *S. anatolicum* chromosome (up to 1.75 seeds/spikelet). However, the position of the gene has to be precise. The allele carried by the *S. anatolicum* genome could be different from the one present on the rye genome. Due to the different genome rearrangements between these two *Secale* species, the *S. anatolicum* chromosome could be easier to translocate into the wheat genome. It is interesting to note that three restorer genes (*Rfp1*, *Rfp2* and *Rfp3*) against rye Pampa (P)-cytoplasm, used for hybrid rye production, have been located on chromosome 4RL in the same region (Hackauf et al., 2017; Stracke et al., 2003). A previous example of a locus

restoring the fertility against different sterile cytoplasms has been reported. Chromosome 1BS of wheat carries a locus involved in the restoration of male fertility in CMS lines with *Ae. kotschyi*, *Ae. mutica* and *Ae. uniaristata* cytoplasm (Tsunewaki, 2015). This locus has been named as Rf^{multi}. In addition to the ability of chromosome 4R to restore *T. timopheevii* cytoplasm, this chromosome carries two other genetic factors involved in pollen production (one of the criteria for breeding the male line in the CMS system). Addition of the long arm of chromosome 4R is associated with an increased anther size of 16% and the short arm of chromosome 4R with an increased pollen grain number of 33% (Nguyen et al., 2015). Further study needs to be done to establish the effect of chromosome 4R of *S. anatolicum* on anther length and pollen grain number.

The combination of GISH/FISH and KASP results were very useful as tools to characterize *S. anatolicum* chromosomes. Whereas KASP genotyping allowed a high throughput analysis, no information was given on the number of copies of the *S. anatolicum* chromosome(s). However, *in situ* hybridization gave information on the precise number of chromosomes of *S. anatolicum* present but some confusion might occur with their characterization.

Surprisingly the lines showing the highest fertility restoration level carried more than one *S. anatolicum* chromosome. For example, line 9 set the highest number of selfed seeds (1.94 seeds/spikelet) and was composed by one complete chromosome (2R) and two telocentric chromosomes of *S. anatolicum* (2RL and 4RL). In comparison lines with only chromosome 4RL produced a maximum of 1.75 seeds/spikelet (e.g. line 8). Some modifier loci, enhancing the fertility restoration, could be present on these extra *S. anatolicum* chromosomes in an analogy with the presence of modifier loci on the wheat genome against *T. timopheevii* cytoplasm (Maan et al., 1984; Robertson and Curtis, 1967; Würschum et al., 2017b). Localization of these modifier loci, either on the wheat or the rye genome, need to be studied in order to maximize fertility restoration. The second explanation for the

difference in the fertility observed could be the higher genome stability of the lines carrying several *S. anatolicum* chromosomes. The work has also shown that restoration by chromosome 4RL was not stable as the fertility ranged from 0 to 1.75 seeds/spikelet. It has been shown that some interaction between the wheat and the wild genome could cause silencing of the gene of interest (Baum et al., 1992) and that aneuploidy causes alteration in pollen viability (Zhang et al., 2013).

Conclusion

This chapter confirmed the presence of a restorer gene against *T. timopheevii* cytoplasm in the genome of *S. anatolicum*. This gene was located on chromosome 4RL and caused partial fertility restoration. However, restoration was unstable as the *Rf* chromosome was transmitted as an addition or a substitution. The *Rf* locus has to be stabilized to enhance the restoration of the fertility before studying it in more detail, e.g. the ability of the restoration in multiple genetic background and environment. Back crossed generations are also required to reconstitute the wheat genome. To fix the genome of *S. anatolicum* in a wheat background, a translocation event has to happen into the wheat genome. Production of fertile translocation lines with smaller *S. anatolicum* fragments will allow a more precise localization of the *Rf* locus as the accuracy of the KASP markers was confirmed. However, other restorer factors might be present in the *S. anatolicum* genome. For example a second restorer gene was located on the distal part of the long arm of chromosome 6R of rye and completely restored fertility (Curtis and Lukaszewski, 1993). The other potential restorer might have been lost through the selfing process or might have been initially missing in the amphidiploid used as the male parent.

V. Chapter 5: Development of wheat/*Secale* introgression lines as potential restorer lines against *T. timopheevii*

Summary

The aim of this chapter was to produce wheat/wild *Secale* species translocation lines with the ultimate purpose of introgressing a restorer factor against *T. timopheevii* cytoplasm. For that purpose, wheat/*S. anatolicum*, wheat/*S. iranicum* and wheat/*S. vavilovii* amphidiploids were pollinated by either Chinese Spring *ph1* mutant or Paragon *ph1* mutant. Back crosses were carried out with not mutant wheat. Genomic *in situ* hybridization techniques were used to characterize the lines produced. No translocation lines were produced but a wide range of wheat/*S. anatolicum* and wheat/*S. iranicum* addition and substitution lines were detected. These lines be used to study more precisely these two *Secale* genome as potential reservoir of traits for wheat improvement.

V.1. Introduction

V.1.1. *Secale* species, a huge reservoir of diversity for wheat improvement

The genus *Secale* is divided into three groups: *S. strictum*, *S. sylvestre* and *S. cereale* (Frederiksen and Petersen, 1998) (refer to the Chapter IV.1.1). Syntenic relationships between the rye and the wheat genomes have been well studied bringing to light several chromosomal rearrangements involving chromosome arms 2RS, 3RL, 4RL, 5RL, 6R, 6RL, 7RS and 7RL (Devos et al., 1993; Li et al., 2013). However, several chromosome rearrangements have also occurred within the *Secale* genus, especially between groups 2, 6 and 7 (Devos et al., 1993; Heemert and Sybenga, 1972; Singh and Röbbelen, 1977; Sybenga et al., 1985). Wheat and *S. montanum* differ by three translocation events involving linkage groups 4L/5L, 4L/7S and 3L/6L (Devos et al., 1993). This genus includes a wide diversity of species by their reproductive and growing habit and thus this genus is considered as a huge reservoir of interesting genes for wheat

improvement (Maraci et al., 2018; Schreiber et al., 2018). It has been shown that important diversity existed within the subspecies in relation with their geographical distribution (Maraci et al., 2018). However, most of the introgression programmes of the *Secale* genome into wheat have been done, until now, only with cultivated rye (Crespo-Herrera et al., 2017; Fu et al., 2014b). For example, the 1BL.1RS translocation which originated from the rye cultivar Pektus, is widely present in wheat cultivars and carries several resistance genes (Friebe et al., 1996). However some of these resistance genes, such as *Lr26* (leaf rust), *Pm8* (powdery mildew), *Sr31* (stem rust) and *Yr9* (yellow rust) are no longer completely effective (Bartos, 1993; Lutz et al., 1992; Pretorius et al., 2000; Rabinovich, 1998). New sources of resistance genes have been searched for in the genomes of the wild *Secale* species and introduced into wheat breeding programme. For example, a potential new allele for stripe rust resistance has been found on the long arm of chromosome $2R^{afr}$ of *S. africanum* (Lei et al., 2011). Another yellow rust resistance gene has been localized on chromosome $6R^{m}$ of *S. montanum* (Schneider et al., 2016).

V.1.2. Introgression of *Secale* genome into wheat

Introgression of wild species genomes into wheat can be achieved via different ways (refer to Chapter I.6). Complete chromosomes can be added to or substituted for wheat chromosomes leading to the formation of addition lines or substitution lines respectively. However, addition of a complete chromosome is often associated with linkage drag. For that reason, translocation of small fragments of the *Secale* genome into the wheat genome has been considered as the most promising material. The transfer of a targeted fragment of *Secale* into the wheat genome can be achieved by different strategies such as spontaneous translocation by using monosomic substitution lines, ionizing irradiation or disabling the *Pairing homoeologous* (*Ph*) system. Only spontaneous translocations (the result of centric breakage-fusion behaviour of univalent during the meiosis) have been reported to date. Consecutive selfing generations, after crossing wheat with a $2R^{afr}$(2D) and a $5R^{afr}$(2D) substitution line, resulted in the production of a number of wheat/*S.*

africanum chromosome $2R^{afr}$ and wheat/*S. africanum* chromosome $5R^{afr}$ translocation lines respectively (Lei et al., 2013; Li et al., 2016a).

V.1.3. Aim and objectives of this chapter

The huge diversity existing among the *Secale* genus leads to the hypothesis that restorer genes, other than the one already detected, are present in the *Secale* genome. However, in order to optimize the detection of new restorer factors, i.e. to overcome the post-zygotic barriers encountered by directly testing the complete *Secale* genome using the amphidiploids (which caused a high rate of sterility), *ph1* mutant wheat was used to induce translocations of small fragments of the genomes of different *Secale* species into wheat. Secondly, the wheat/*Secale* introgression lines produced were scored for their ability to restore *T. timopheevii* cytoplasm.

V.2. Materiel and Methods

V.2.1. Plant material

Seven different amphidiploids were used as initial parents: five wheat/*S. anatolicum*, one wheat/*S. vavilovii* and one wheat/*S. iranicum* amphidiploids (**Table V-1**). The details of the production of these amphidiploids is described in Chapter 3. These amphidiploids were crossed with *ph1* mutant wheat, either Paragon or Chinese Spring (CS), with the *Ph1* locus deleted. Back crosses were carried out with pollen of normal wheat (Paragon and Chinese Spring). Two CMS lines with the cytoplasm of *T. timopheevii*, Atomo and Arturnick, both spring type, were used as the female line to test the restoration ability of the introgression lines.

Table V-1 Pedigree of the wheat/*Secale* amphidiploids used as initial parent

Amphidiploid	Wheat parent	Wild parent
1/6/2	CS	*S. anatolicum P208/141*
94/1	Pavon 76	*S. anatolicum P208/141*
5/8/7	CS	*S. anatolicum P208/142*
95/6/4	Highbury	*S. anatolicum P208/142*
99/11/8	CS	*S. anatolicum P208/142*
92/1/9	Pavon 76	*S. iranicum P208/11*
90/4/6	CS	*S. vavilovii 573649*

V.2.2. Crossing program

Figure V-1 illustrates the crossing scheme chosen to develop wheat/*Secale* translocation lines and test their ability to restore *T. timopheevii* cytoplasm. The initial cross was carried out by reciprocally crossing the wheat/*Secale* amphidiploid with wheat *ph1* mutant and also reciprocally (refer to Chapter II.1.4). F_1 plants were also pollinated with *ph1* mutant and then back-cross generations were produced with the pollen of non-*ph1* mutant wheat. BC_1 plant pollen was used to pollinate CMS lines. F_1 plants resulting from these crosses were scored for their fertility level.

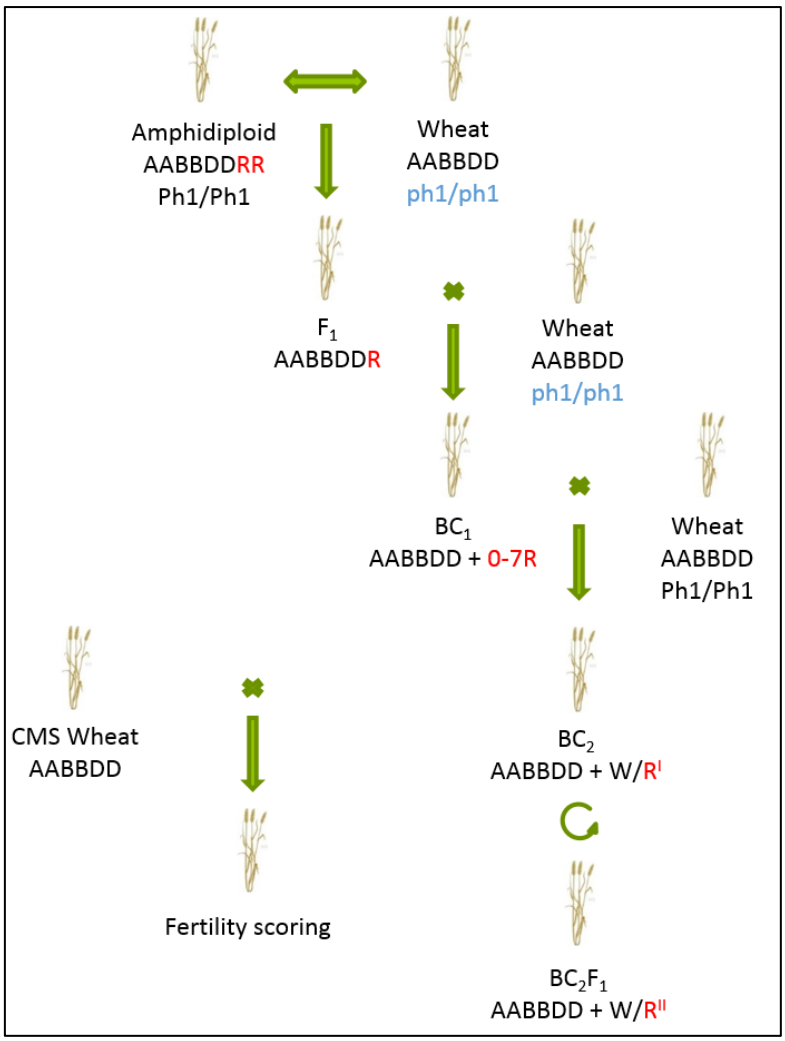


Figure V-1 Crossing scheme to produce wheat/*Secale* translocation lines, potential restorer of *T. timopheevii* cytoplasm
W/R^{I} means a wheat/*Secale* translocated chromosome in heterozygous stage, W/R^{II} means a wheat/*Secale* translocated chromosome in homozygous stage

V.2.3. Seed germination

Germination of the seeds was carried out according to the protocol (refer to Chapter II.1.1). Once the seed germinated, it was sown into soil (John Innes compost No. 2). Amphidiploids were put into vernalisation for eight weeks (at 8°C with 12 hours light per day), F_1 generation plants for six weeks, wheat varieties and the advanced generation lines for four weeks. Plants were grown in the glasshouse (at 21°C, 16 hours light per day).

V.2.4. Cytogenetic analysis

Preparation of metaphase spreads was as described in Chapter II.3.3. Slides were probed as follows. Multi-colour GISH was carried out using total genomic DNA of *Ae. speltoides* as blocking DNA and total genomic DNA of the *Secale* species labelled by nick translation with Alexa Fluor 546-14-dUTP (Invitrogen; C11401) (gold), total genomic DNA of *T. urartu* labelled by nick translation with Alexa Fluor 488-5-dUTP (Invitrogen; C11397) (green) and total genomic DNA of *Ae. tauschii* labelled with Alexa Fluor 594-5-dUTP (Invitrogen; C11400) (red) were used as probe.

V.2.5. Assessment of the success of the crosses

Various observations for each head were done to determine the success of the crosses:

- Percentage of fertile heads = (Number of crossed heads setting seeds)/(Total number of crossed heads) *100
- Percentage of plants setting crossed seeds = (Number of crossed plants setting seeds)/(Total number of crossed plants) *100
- Average number of seeds per crossed head = (Total number of seeds)/(Total number of crossed heads)
- Percentage of shrivelled seeds = (Number of shrivelled seeds)/(Total number of seeds) *100
- Percentage of germination = (Number of seeds germinated)/(Total number of seeds sown) *100
- Percentage of self-fertile plants = (Number of plants setting selfed seeds)/(Number total of plants)*100
- Average number of selfed seeds per head = (Total number of selfed seeds)/(Total number of selfed heads)

V.2.6. Fertility restoration scoring

To score the male fertility restoration, heads of the F_1, resulting from the pollination of the CMS line by the BC_1 lines, were bagged prior to anthesis and

harvested after ripening. For each selfed spike, number of seeds as well as the number of spikelets was counted and both were averaged by plant. The seed set of a plant was defined as the number of seeds divided by the number of spikelets. A plant without any selfed seed was considered as male sterile, whereas a plant with at least one seed was considered as male fertile.

V.3. Results

V.3.1. Crosses results

V.3.1.1. *Production of the F_1 generation*

A total of 421 heads were crossed and 479 F_1 seeds were produced. The comparison of the reciprocal crosses between wheat *ph1* mutant and *Secale* amphidiploids is presented in **Table V-2**.

Table V-2 Results of the reciprocal crosses between Wheat *ph1* mutant and *Secale* amphidiploid

Wild species	Observation	Amphidiploid x Wheat	Wheat x Amphidiploid
S. anatolicum	Percentage of fertile crossed heads	36.53% (99/271)	19.48% (15/77)
	Average number of seeds per crossed head	1.29 (350/271)	0.48 (37/77)
	Percentage of shrivelled seeds	22.29% (78/350)	81.08% (30/37)
S. iranicum	Percentage of fertile crossed heads	56.10% (23/41)	54.55% (6/11)
	No. of seeds per crossed head	1.49 (61/41)	2.45 (27/11)
	Percentage of shrivelled seeds	45.90% (28/61)	100% (27/27)
S. vavilovii	Percentage of fertile crossed heads	16.67% (3/18)	33.33% (1/3)
	Average number of seeds per crossed head	0.17 (3/18)	0.33 (1/3)
	Percentage of shrivelled seeds	100% (3/3)	100% (1/1)

V.3.1.2. *Back crossed generation*

F_1 resulting from crosses between *Secale* amphidiploids as female parent and wheat *ph1* mutant as male were backcrossed using wheat pollen (**Table V-3**).

Table V-3 Results crosses for each *Secale* species for the different generations

Wild species	Observations	F_1	BC_1	BC_2
S. anatolicum	Percentage of germination	73.07% (57/78)	91.53% (54/59)	95.45% (42/44)
	Percentage of crossed plants setting seeds	82.35% (42/51)	100% (40/40)	90.91% (10/11)
	Number of seeds per head crossed	2.73 (318/134)	7.04 (1317/187)	7.75 (155/20)
	Percentage of self-fertile plants	54.05% (20/37)	86.84% (33/38)	84.61% (33/39)
	Number of selfed seeds per head	6.41 (237/37)	11.48 (459/40)	14.40 (605/42)
S. iranicum	Percentage of germination	91.67% (11/12)	86.67% (13/15)	100% (12/12)
	Percentage of crossed plants setting seeds	70% (7/10)	100% (8/8)	100% (4/4)
	Number of seeds per head crossed	1.71 (36/21)	5.67 (221/39)	14.38 (115/8)
	Percentage of self-fertile plants	28.57% (2/7)	57.14% (4/7)	91.67% (11/12)
	Number of selfed seeds per head	0.57 (4/7)	5.71 (10/7)	14.23 (185/13)
S. vavilovii	Percentage of germination	66.66% (2/3)		
	Percentage of crossed plants setting seeds	50% (1/2)		
	Number of seeds per head crossed	0.13 (1/8)		
	Percentage of self-fertile plants	0% (0/2)		
	Number of selfed seeds per head	0 (0/2)		

V.3.2. Characterization by Genomic *In Situ* Hybridization

V.3.2.1. *Genomic constitution of the Secale amphidiploids*

The genomic composition of the amphidiploids used as the initial parent was determined using multi-colour GISH to distinguish the chromosomes of the A, B, D and R genomes (**Table V-4** and **Figure V-2**). Variable compositions of the

A, D and R genomes were found in the different amphidiploids, ranging from 8 to 15 chromosomes by genome while the B-genome composition was stable with a total number of 14 chromosomes. For example, one *S. anatolicum* amphidiploid 95/6/4 was composed of 15 A-genome chromosomes, 14 B-genome chromosomes, 14 D-genome chromosomes and 14 *S. anatolicum* chromosomes (**Figure V-2 A**). Another *S. anatolicum* amphidiploid 94/1 was composed of 14 A-genome chromosomes, 14 B-genome chromosomes, 12 D-genome chromosomes and 9 *S. anatolicum* chromosomes (**Figure V-2 B**).

Table V-4 Different genomic compositions of the amphidiploid used as parent
3*0.5 means that three telocentric chromosomes are present

Amphidiploid	A-genome	B-genome	D-genome	R-genome
1/6/2	13	14	13	13
1/6/2	14	14	14	11
1/6/2	14	14	13	12
92/1/9	14	14	14	10
94/1	14	14	12	10
94/1	14	14	12	9
94/1	14	14	12	11
94/1	14	14	12	7 + 3*0.5
94/1	14	14	12	8
95/6/4	14	14	14	10
95/6/4	14	14	14	14
95/6/4	15	14	14	14

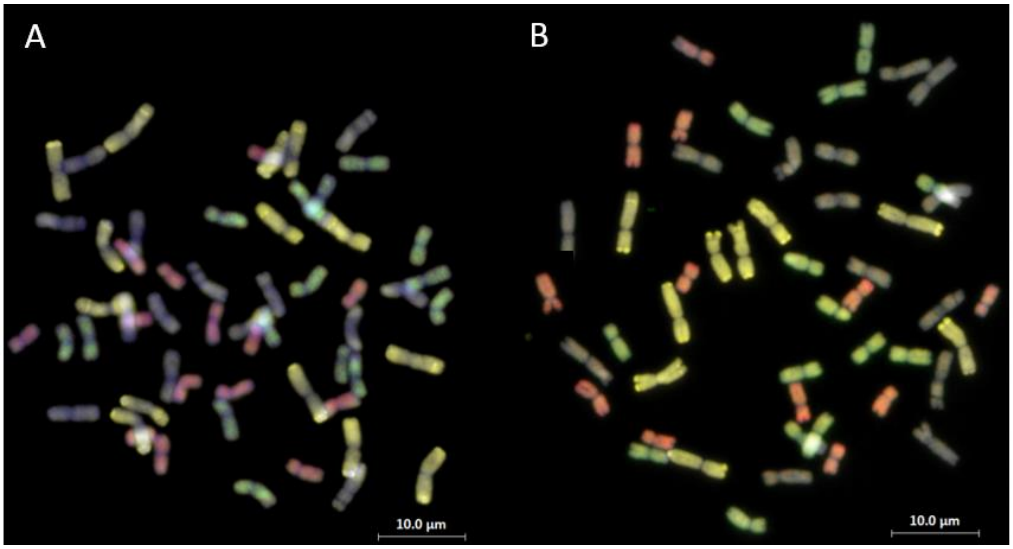


Figure V-2 Multicolour GISH image of two *Secale* amphidiploids
A genome in green, B genome in blue, D genome in red and *Secale* genome in gold. A) 95/6/4 amphidiploid composed by 15A + 14B + 14D + 14R chromosomes. B) 94/1 amphidiploid composed by 14A + 14B + 12D + 9R chromosomes

V.3.2.2. *Genomic constitution of the wheat parents*

The genomic composition of the wheat used as the male parent in the crossing programme was also determined by using multi-colour GISH (**Table V-5** and **Figure V-3**). Chinese Spring, Paragon (**Figure V-3 A**) and Paragon *ph1* mutant (**Figure V-3 B**) had 14 chromosomes of each genome (i.e. A-genome, B-genome and D-genome) with a total of 42 chromosomes. However, the genomic composition of CS *ph1* mutant was highly variable (**Table V-5** and **Figure V-3 C** and **D**). Fourteen A-genome chromosomes were present, whereas the chromosome number of the B- and D-genome varied between 12 to 16 and 14 to 17 respectively. CS *ph1* mutant carried 42 to 45 chromosomes in total.

Table V-5 Genomic composition of the different wheat genotypes used as recurrent parents

Wheat	A-genome	B-genome	D-genome	Total
CS	14	14	14	42
Paragon	14	14	14	42
CS *ph1* mutant	14	14	14	42
CS *ph1* mutant	14	14	15	43
CS *ph1* mutant	14	14	17	45
CS *ph1* mutant	14	16	14	44
CS *ph1* mutant	14	12	16	42
CS *ph1* mutant	14	15	14	43
Paragon *ph1* mutant	14	14	14	42

In addition, translocations within the wheat chromosomes were present in both CS *ph1* mutant and Paragon *ph1* mutant. **Figure V-3 B** shows a metaphase of a cell of Paragon *ph1* mutant with a small telomeric reciprocal translocation between the A and the D genomes. **Figure V-3 C** shows a metaphase of a cell of CS *ph1* mutant with a small telomeric homozygous translocation of the D genome into the A genome and a larger homozygous translocation between the B and the D genomes. **Figure V-3 D** shows a metaphase of a cell of CS *ph1* mutant with a small telomeric reciprocal translocation between the A and the D genomes and a larger single translocation between the A and the D genomes.

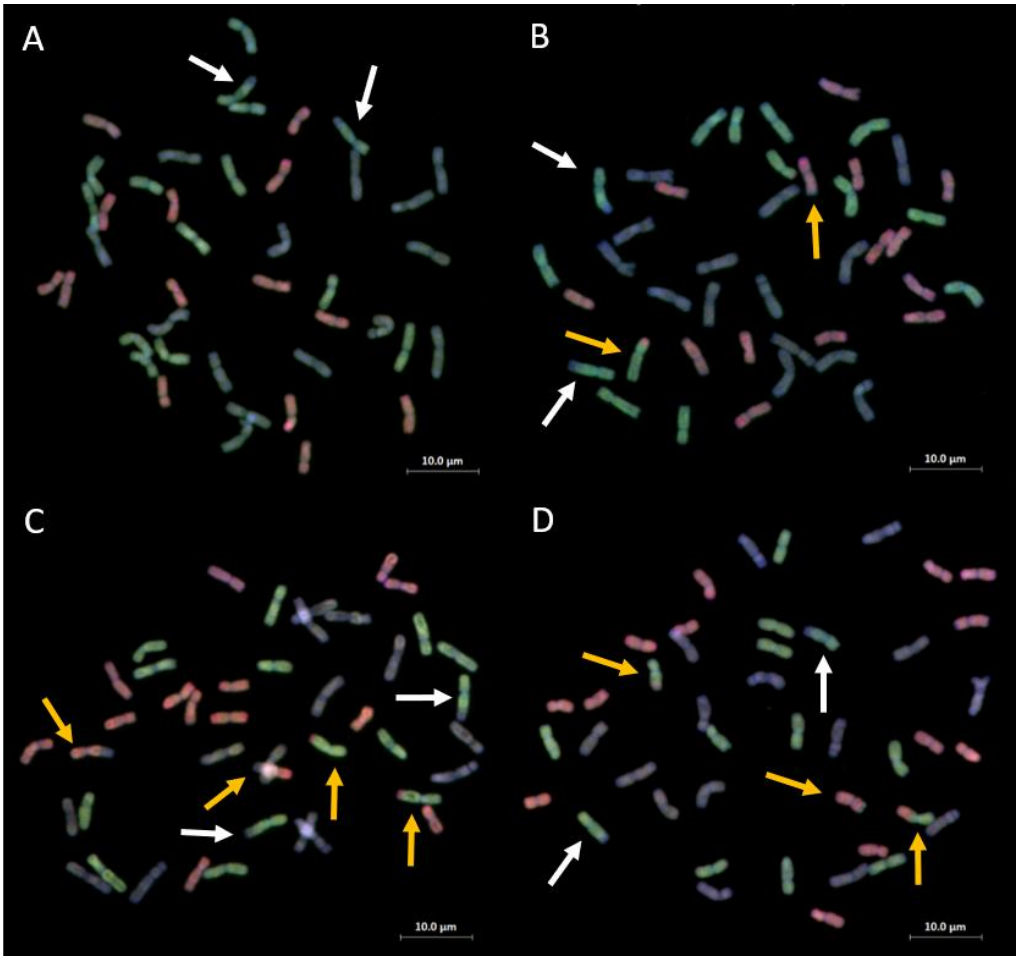


Figure V-3 Multicolour GISH image of wheat parents

A genome in green, B genome in blue, D genome in red. White arrows indicate the 4AL/7BS translocation. Yellow arrows indicate translocations within the wheat genomes. A) Paragon composed by 14A + 14B + 14D chromosomes. B) Paragon *ph1* mutant composed by 14A + 14B + 14 D chromosomes. C) CS *ph1* mutant composed by 14A + 14B + 15D chromosomes. D) CS *ph1* mutant composed by 14A + 15B + 15D chromosomes

V.3.2.3. *Genomic constitution of the back crossed lines*

V.3.2.3.1. BC_1 generation

Multi-colour GISH was successfully carried out on 66 of the 67 BC_1 plants grown. 49 lines were detected as carrying chromosomes of the *Secale* genome. Added or substituted telocentric or complete chromosomes were detected but no translocations of the *Secale* genome into the wheat genome were found (**Figure V-4**). BC_1 lines contained between 0 (22.22%) and 5 complete chromosomes (5.56%) of *S. anatolicum* and 0 (41.67%) and 4 chromosomes (8.33%) of *S. iranicum*.

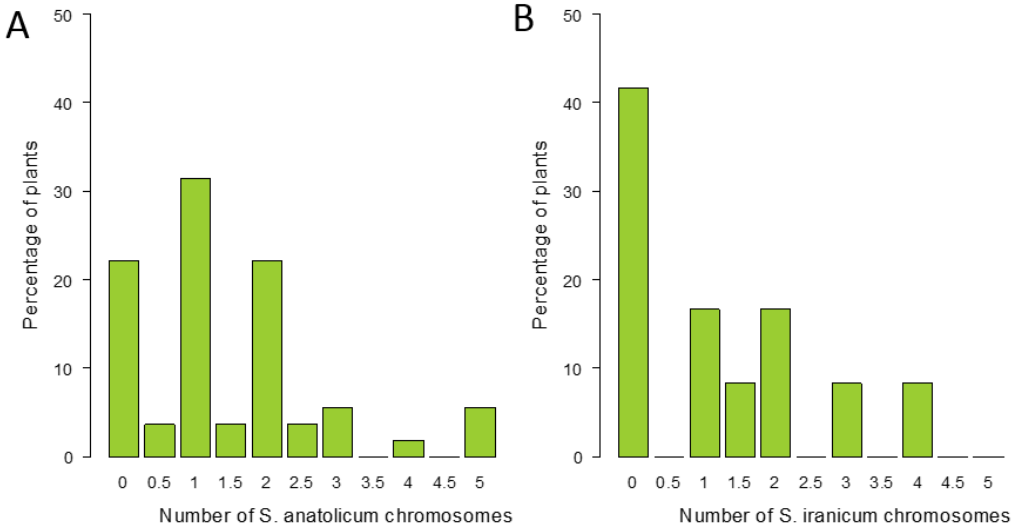


Figure V-4 Distribution of the number of chromosomes of A) *S. anatolicum* and B) *S. iranicum* present in the BC_1 lines

The quality of the GISH images allowed the characterization of the total genome composition of 47 lines (A, B, D and R genomes) (**Table V-6**).

Table V-6 Classification of the BC_1 lines produces according to their number of wheat and *Secale* chromosomes

Designation	**Composition**	**No. of *S. anatolicum* BC_1 lines**	**No. of *S. iranicum* BC_1 lines**
Monosomic substitution	39W+ 1R	1	0
Monotelosomic substitution	41W + 0.5R	1	0
Monosomic substitution	41W + 1R	5	1
Monotelosomic addition	42W + 0.5R	1	0
Monosomic addition	42W + 1R	10	1
Multisomic addition	42W + (1.5-5) R	11	3
Other		12	1

- Substitution lines

A total of seven wheat/*S. anatolicum* substitution lines were produced: one monotelosomic substitution line (41W + 0.5R), one monosomic substitution line with three wheat chromosomes missing (39W + 1R) and five monosomic substitution lines (41W + 1R) (**Table V-6** and **Figure V-5**). One monosomic

wheat/*S. iranicum* line was produced. Despite a total wheat chromosomes number of 42, some wheat genomes were unbalanced with a chromosome of one genome missing and one extra chromosome of another genome present. **Figure V-5 B** shows a multi-colour GISH image of a monosomic substitution line with 15 A-genome chromosomes, 13 B-genome chromosomes and 14 D-genome chromosomes. This line also carried a small homozygous telomeric translocation of the D genome into the A genome of wheat.

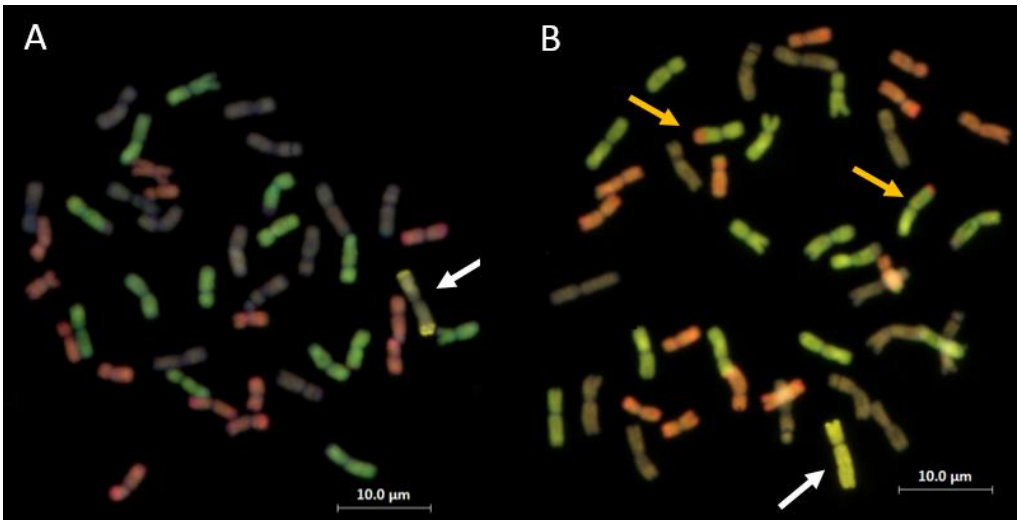


Figure V-5 Multicolour GISH image of two BC_1 monosomic substitution wheat/*S. anatolicum* lines

A genome in green, B genome in blue, D genome in red and R genome in gold indicated by the white arrows. Yellow arrows indicate translocations within the wheat genomes. A) Line composed by 14A + 13B + 14D + 1R chromosomes. B) Line composed by 15A + 13B + 13D + 1R

- Addition lines

A total of 22 wheat/*S. anatolicum* addition lines were produced, i.e. 42 wheat chromosomes to which was added between a half to 5 *S. anatolicum* chromosomes (**Table V-6**). One of these lines was a monotelosomic addition line (half chromosome of *S. anatolicum*), ten lines were revealed to be monosomic addition lines (1 complete chromosome of *S. anatolicum*) (**Figure V-6**) and the others were multisomic addition lines (**Figure V-7**). The line with the highest chromosome number (47 chromosomes) contained 5 *S. anatolicum* chromosomes. Out the 22 addition lines, only 19 had the usual wheat genome composition, i.e. 14 A-genome chromosomes, 14 B-genome

chromosomes and 14 D-genome chromosomes (**Figure V-6 A** and **Figure V-7 A). Figure V-7 A** highlights the presence of a small telomeric homozygous translocation of the D genome into the A genome of wheat and a single translocation of the A-genome segment in a D-genome chromosome in an addition line with 13 A-genome chromosomes, 14 B-genome chromosomes and 15 D-genome chromosomes. Moreover, three small translocations of the D genome into the A genome were detected. **Figure V-7 B** shows a monosomic addition line with 14 A-genome chromosome, 15 B-genome chromosomes and 13 D-genome chromosomes. Other lines missed one chromosome of one genome and had one extra chromosome of another genome. For example, **Figure V-6 B** shows a monosomic addition line with 13 A-genome chromosomes, 14 B-genome chromosomes and 15 D-genome chromosomes. Moreover, three small translocations of the D genome into the A genome were detected. **Figure V-7 B** shows a monosomic addition line with 14 A-genome chromosome, 15 B-genome chromosomes and 13 D-genome chromosomes.

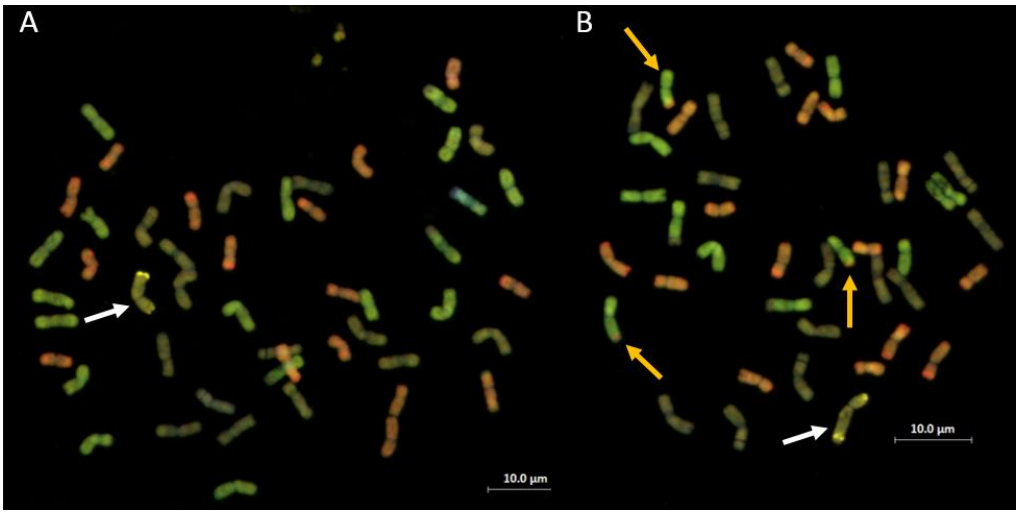


Figure V-6 Multicolour GISH image of two BC_1 monosomic addition wheat/*S. anatolicum* lines

A genome in green, B genome in blue, D genome in red and R genome in gold indicated by the white arrows. A) Line composed by 14A + 14B + 14D + 1R chromosomes. B) Line composed by 13A + 14B + 15D + 1R chromosomes

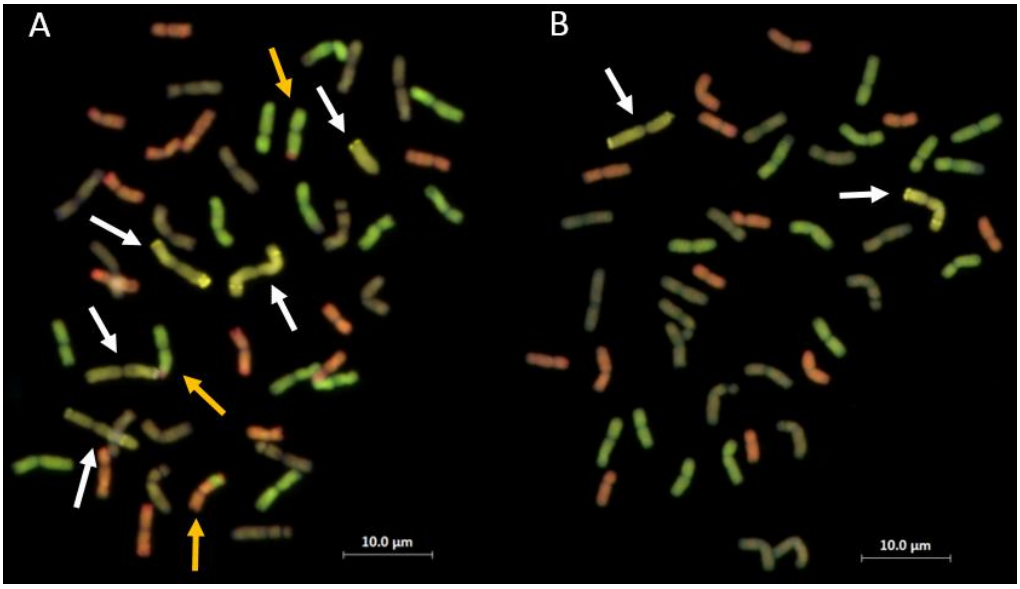


Figure V-7 Multicolour GISH image of two BC_1 multisomic addition wheat/*S. anatolicum* lines

A genome in green, B genome in blue, D genome in red and R genome in gold indicated by the white arrows. Yellow arrows indicate translocations within the wheat genomes. A) Line composed by 14A + 14B + 14D + 4.5R chromosomes. B) Line composed by 14A + 15B + 13D + 2R chromosomes

A total of four wheat/*S. iranicum* addition lines were produced: one monosomic (**Figure V-8 A**) and 3 multisomic addition lines (between 2 and 4 chromosomes of *S. iranicum*) (**Figure V-8 B** and **Table V-6**). **Figure V-8 B** shows the presence of a small telomeric homozygous translocation of the A genome into the D genome of wheat and a larger telomeric translocation of the D genome into the A genome of wheat.

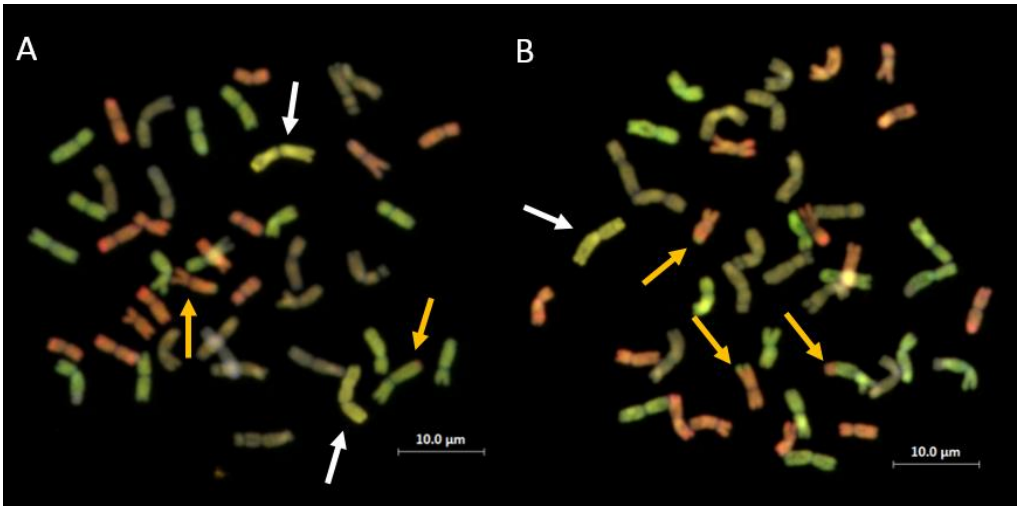


Figure V-8 Multicolour GISH image of two BC_1 addition wheat/*S. iranicum* lines

A genome in green, B genome in blue, D genome in red and R genome in gold indicated by the white arrows. Yellow arrows indicate translocations within the wheat genomes. A) Line composed by 14A + 14B + 14D + 2R chromosomes. B) Line composed by 14A + 13B + 15D + 1R chromosomes.

- Other lines

Thirteen lines were detected as carrying a number of wheat chromosomes which differed from the normal 42 by having a total number of chromosomes above this number (**Table V-6**). These plants were composed of between 12 to 16 chromosomes of each wheat genome and by 1 to 5 chromosomes of *Secale*, leading to a total chromosome number between 42.5 and 46. For example, **Figure V-9 A** shows the metaphase of a cell with 14 A-genome chromosomes, 14 B-genome chromosomes, 15 D-genome chromosomes and 1 chromosome of *S. anatolicum,* whereas **Figure V-9 B** shows the metaphase of a cell with 14 A-genome chromosomes, 12 B-genome chromosomes, 12 D-genome chromosomes and 4 complete chromosomes of *S. anatolicum* plus two chromosome arms of *S. anatolicum*.

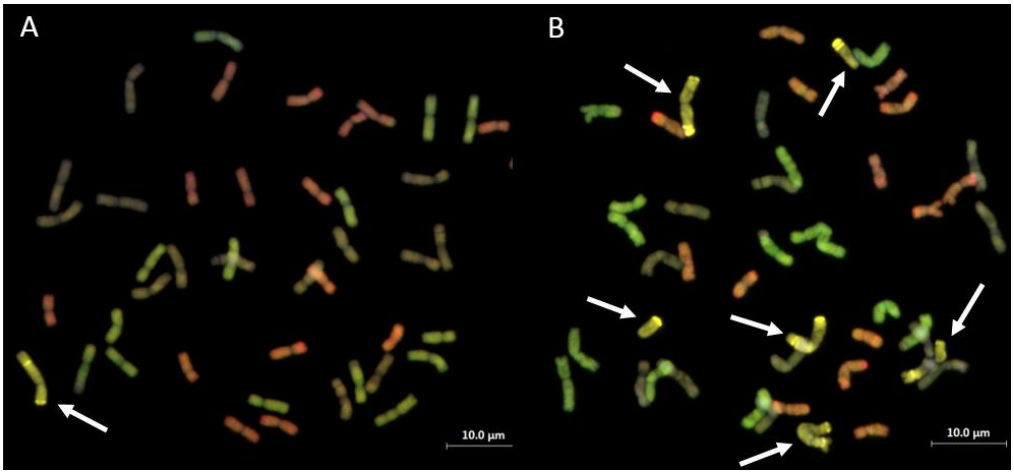


Figure V-9 Multicolour GISH image of two BC_1 introgression wheat/*S. anatolicum* lines

A genome in green, B genome in blue, D genome in red and R genome in gold indicated by the white arrows. A) Line composed by 14A + 14B + 15D + 1R chromosomes. B) Line composed by 14A + 12B + 12D + 4 complete and two telocentric R chromosomes

V.3.2.3.2. BC_2 generation

Out of the 54 BC_2 plants grown in the glasshouse, 15 lines were detected as carriers of regions of the *Secale* genome. Telocentric or complete chromosomes of *Secale* added or substituted were detected but no translocations of the *Secale* genome into the wheat genome were found. 73.81% of the BC_2 lines lost the *S. anatolicum* chromosomes, 9.52% retained a telocentric chromosome and 16.67% a single complete chromosome (**Table V-7**). 66.67% of the BC_2 lines lost the *S. iranicum* chromosomes and 33.33% retained a single complete chromosome.

Table V-7 Percentage of BC_2 lines carrying none, telocentric or a complete chromosome o *S. anatolicum* or *S. iranicum*

No. of *Secale* chromosome	*S. anatolicum* BC_2 lines	*S. iranicum* BC_2 lines
0	73.81%	66.67%
0.5	9.52%	0%
1	16.67%	33.33%

The quality of the GISH images allowed the characterization of the genome composition of 10 lines: 5 of them were characterized as monosomic addition

lines and 5 of them as substitution lines (**Table V-8**). **Figure V-10 A** shows a metaphase of a cell of a monosomic addition line with 14 chromosomes of each genome of wheat and one complete chromosome of *S. anatolicum*. **Figure V-10 B** shows a metaphase of a cell of a monosomic substitution line with one chromosome of the D-genome of wheat substituted by one chromosome of *S. anatolicum*.

Table V-8 Classification of the BC_2 lines produces according to their number of wheat and *Secale* chromosomes

Designation	**Composition**	**No. of *S. anatolicum* BC_2 lines**	**No. of *S. iranicum* BC_2 lines**
Monotelosomic substitution	41W + 0.5R	2	0
Monosomic substitution	41W + 1R	3	0
Monosomic addition	42W + 1R	3	2

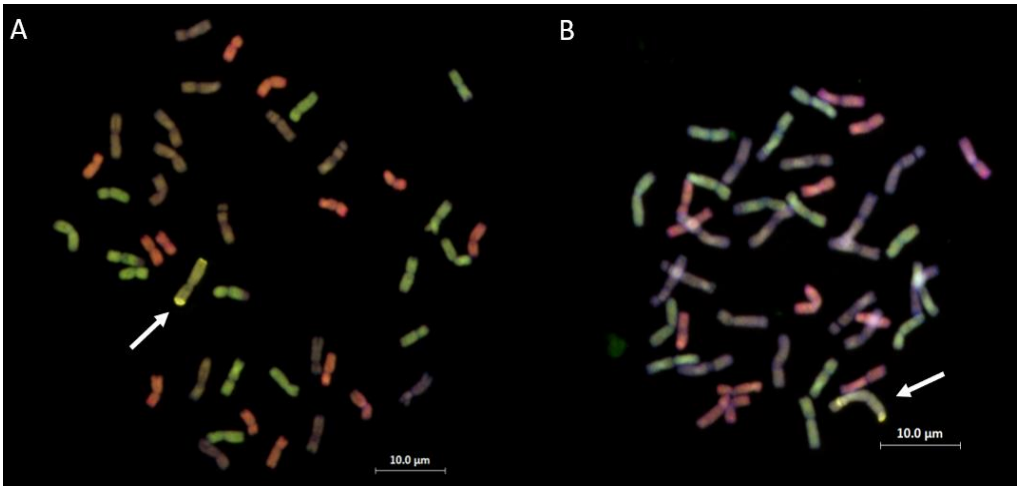


Figure V-10 Multicolour GISH image of two BC_2 lines
A genome in green, B genome in blue, D genome in red and R genome in gold indicated by the white arrows. A) Wheat/*S. anatolicum* addition line composed by 14A + 14B + 14D + 1R chromosomes. B) Wheat*/S. anatolicum* monosomic substitution line composed by 14A + 14B + 13D + 1R chromosomes.

V.3.2.3.3. Transmission of the *Secale* chromosomes through the generations

Only 15 out the 54 BC_2 plant germinated (28%) carried any *Secale* chromosomes. **Figure V-11** shows the percentage of transmission of the *Secale*

genome from the BC_1 generation to the BC_2 generation. 85.71%, 66.67%, 83.33%, 64.29% of the BC_2 plants (offspring of the BC_1 plants with a telocentric, one whole, one whole and half, two *Secale* chromosomes) respectively, completely lost the *Secale* genome. A telocentric chromosome present in the BC_1 plants was transmitted to the offspring in 14.29% of the cases. 28.57% of the offspring of the BC_1 plants carrying a complete *Secale* chromosome carried this chromosome, while 4.76% of the BC_2 plants had only one arm of this *Secale* chromosome. When the BC_1 parent had one complete chromosome and a telocentric chromosome of *Secale*, 8.33% of the BC_2 offspring carried a whole chromosome, while 8.33% carried a telocentric chromosome. One of the two complete *Secale* chromosomes present in the BC_1 generation was transmitted to the offspring in 28.57% of cases, whereas 7.14% of the BC_2 plants carried only one arm of one of these chromosomes.

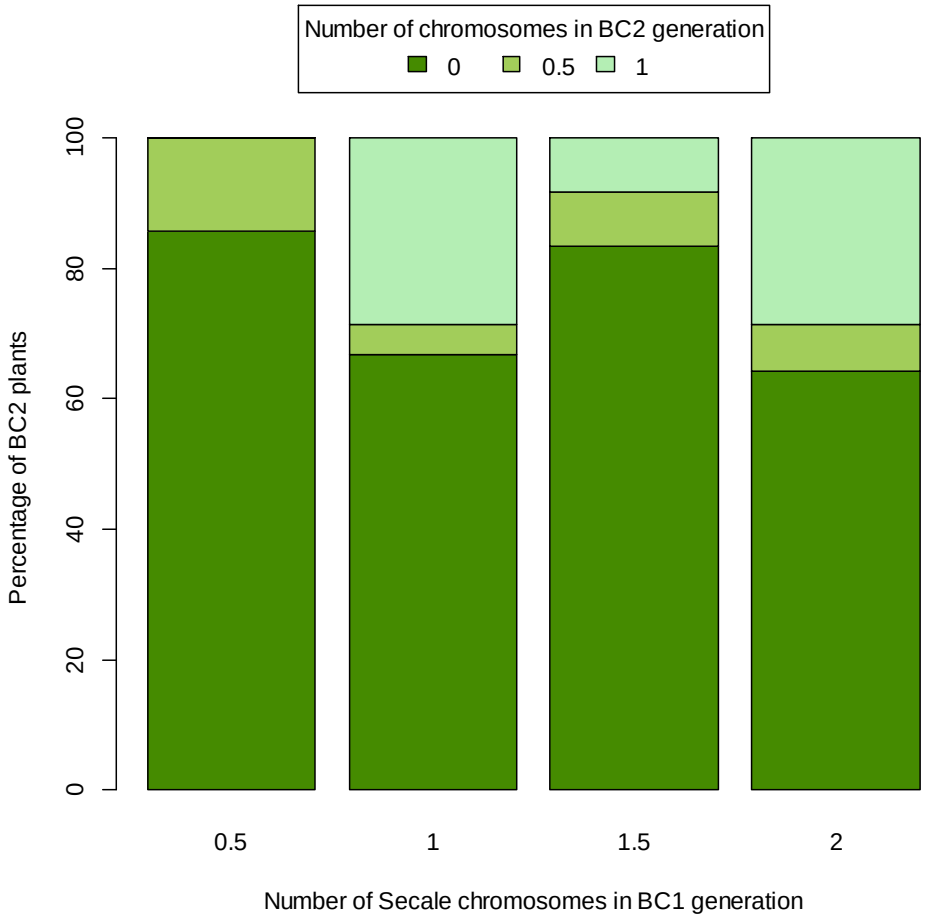


Figure V-11 Transmission of the *Secale* genome from the BC_1 to the BC_2 generation depending of the number of *Secale* chromosomes present in the BC_1 generation

V.3.2.3.4. Self-fertility of the BC_2 generation in relation with the GISH results

Figure V-12 represented the fertility level of the BC_2 generation in relation with the presence or absence of the *Secale* genome. BC_2 plants without *Secale* chromosomes had an average fertility of 0.91 seeds/spikelet with a minimum of 0 and a maximum of 2.32 seeds/spikelet. BC_2 plants with a telocentric *Secale* chromosome set an average of 0.43 seeds/spikelet (minimum 0 and maximum 1.41 seeds/spikelet). However, BC_2 plants carrying a complete *Secale* chromosome showed an average fertility of 0.60 seeds/spikelet with a minimum of 0 and a maximum of 1.95 seeds/spikelet.

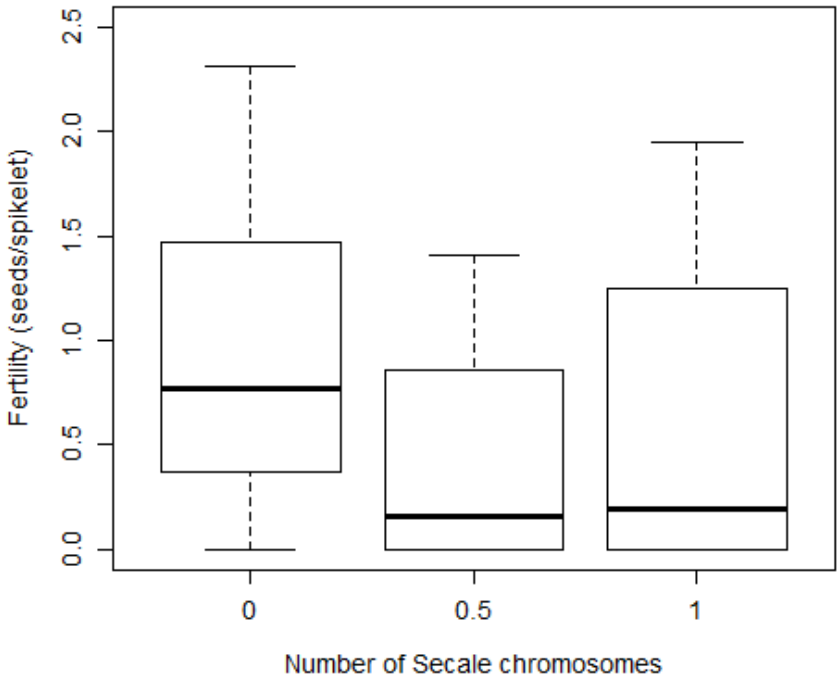


Figure V-12 Distribution of the fertility of the BC_2 generation in regard to the number of *Secale* chromosomes

In the box plots, the boundary of the box closest to zero indicates the 25th percentile, a black line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles.

V.3.3. Fertility restoration ability of the BC_1 lines

V.3.3.1. *Production of the F_1 generation*

BC_1 lines with one complete *Secale* chromosome were used to pollinate the CMS lines. A total of 72 crosses were made, resulting in the production of 225 F_1 seeds (223 seeds for *S. anatolicum* and 2 seeds for *S. iranicum*) (**Table V-9**).

A total of 118 F_1 seeds from *S. anatolicum* BC_1 lines crossed with the CMS lines were put to germinate and 100 F_1 seeds germinated (85%). The two F_1 seeds produced from the *S. iranicum* BC_1 lines also successfully germinated.

Table V-9 Results of the crosses between CMS lines and BC_1 lines with a single chromosome of *S. anatolicum* or *S. iranicum*

	***S. anatolicum* derived line**	***S. iranicum* derived line**
No. of head crossed	63	9
No. of F_1 seeds	223	2
% of germination	80% (100/118)	100% (2/2)

V.3.3.2. *Genomic in situ Hybridization results*

GISH results are presented in **Table V-10**. Out of the 100 F_1 plants derived from the *S. anatolicum* BC_1 lines, 78 lost the *S. anatolicum* chromosome. The whole *S. anatolicum* chromosome was maintained in only 5 of the F_1 lines whereas 18 of the F_1 carried one arm of this chromosome. One translocation line was detected. One F_1 derived from *S. iranicum* BC_1 lines had the complete chromosome, whereas the second line carried a telocentric chromosome.

Table V-10 Genomic *in situ* results of the F_1 generation resulting from the crosses between CMS lines and BC_1 *Secale*/wheat introgression lines

	S. anatolicum	***S. iranicum***
% of F_1 without *Secale* chromosomes	78.00% (78/100)	0% (0/2)
% of F_1 with 1 complete *Secale* chromosome	5.00% (5/100)	50% (1/2)
% of F_1 with telocentric *Secale* chromosome	16.00% (16/100)	50% (1/2)
% of F_1 with translocation of *Secale* chromosome into wheat	1% (1/100)	0% (0/2)

V.3.3.3. *Fertility scoring*

All 102 F_1 lines, with and without the *Secale* genome, were sterile (**Table V-11**).

Table V-11 Percentage of F_1 fertile plants

	Percentage of fertile plants
F_1 without *S. anatolicum* genome	0%
F_1 with *S. anatolicum* genome	0%
F_1 without *S. iranicum* genome	0%
F_1 with *S. iranicum* genome	0%

V.4. Discussion

The aim of this chapter was to produce wheat/wild *Secale* species translocation lines with the ultimate purpose of introgressing a restorer factor against *T. timopheevii* cytoplasm. Two restorer genes have already been localized on the cultivated rye genome: *Rfc3* on chromosome 6RL and *Rfc4* on chromosome 4RL (Curtis and Lukaszewski, 1993). However, regarding the diversity within the genus *Secale* (Maraci et al., 2018), the hypothesis was made that possibly other restorer factors might be present on the wild *Secale* species genomes. In Chapter 4, one *Rf* gene was shown to be located on chromosome 4RL of *S. anatolicum*. The first step was the production of wheat/*Secale* translocation lines. For this purpose, wheat/*Secale* amphidiploids were preferred to the wild *Secale* accession according to the crossing strategy proposed by O'mara (1940). Three different *Secale* species were selected: *S. anatolicum*, S*. iranicum*, and *S. vavilovii*. According to molecular studies, *S. vavilovii*, a short annual plant, was ranked as a subspecies within the cereale group (Hagenblad et al., 2016) despite morphological differences (Khush, 1962). *S. iranicum* is a wild annual, morphologically similar to the *S. cereale* wild species, and in particular to *S. vavilovii* from which its genome differed by a single translocation (Lupton, 1987). *S. iranicum* has been included in the *S. cereale* species according to the most recent classification (Frederiksen and Petersen, 1998). *S. anatolicum*, a perennial from Turkey and western Iran, has been considered as a subspecies of the *S. strictum* species

(Frederiksen and Petersen, 1998) and differed from the *S. cereale* species by two translocations involving three chromosomes (Khush, 1962; Khush and Stebbins, 1961; Krüger, 1947; Riley, 1955; Stutz, 1957), while introgression work has been carried out using *S. cereale,* but until now no work has been done with these three subspecies. In a normal meiosis process, no pairing would occur between the chromosomes of the different genomes of the wheat/rye hybrid because the chromosomes are not homologous. Sometimes, a spontaneous translocation between the wheat and rye genomes occurs, especially in a substitution line, based on the tendency of univalent chromosomes to break during meiosis and form an interchanged chromosome having one entire arm from each of the two parental univalents. It has been shown that the pairing between homoeologous genomes is very rare because of the presence of the *Pairing homoeologous 1* (*Ph1*) dominant locus located on chromosome 5BL of common wheat (Okamoto, 1957; Riley and Chapman, 1958; Sears and Okamoto, 1958) (refer to Chapter I.8.1). It has also been shown that the absence of chromosome 5B, induced between two and five times more pairing between wheat and rye chromosomes (Miller et al., 1994), a paring level which was increased in the presence of the 5BS chromosome arm carrying a pairing promotor (Naranjo et al., 1988). To increase the probability to obtain wheat/*Secale* translocations, *ph1* mutant lines were used as initial parents, i.e. Chinese Spring *ph1* mutant and Paragon *ph1* mutant. The Chinese Spring *ph1* mutant has been a deletion mutant used worldwide in breeding programs to introgress diversity from wild relatives into wheat: (Sears, 1977). Moreover, Chinese Spring has the recessive alleles *kr1* and *kr2* showing higher crossability with rye (Riley and Chapman, 1967). Paragon was considered as an elite variety in the UK (Angus et al., 2011) and was chosen as a donor of good agronomic traits to the lines produced.

F_1 generation production

Reciprocal crosses between the wheat/*Secale* amphidiploid and wheat *ph1* mutant were made in order to produce the F_1 generation. While the seed set was not dependant on the direction of the crosses, the percentage of F_1

shrivelled seeds was drastically reduced by using the amphidiploid as the female parent (by a factor of two for the *S. iranicum* amphidiploid and by a factor of 3.7 for the *S. anatolicum* amphidiploid). These results confirmed the conclusion of the Chapter 3. The two parental lines had different ploidy levels: the *ph1* mutant line was hexaploid (AABBDD) and the wheat/*Secale* amphidiploid was an octoploid (AABBDDRR). Use of the lower ploidy level as the female, .i.e. the *ph1* mutant line, caused the highest frequency of shrivelled seeds (Thompson, 1930). Only the F_1 seeds resulting from the pollination of the wheat/*Secale* amphidiploid by the *ph1* mutant line were used to continue the crossing programme. Success of the crosses, i.e. germination rate, seed set and level of self-fertility, increased through the back-crossed generations with normal wheat as the recurrent parent. This could be explained by the increase in genomic stability through the generations with a reduction of the number of *Secale* chromosomes. Theoretically, the F_1 was composed of 42 wheat chromosomes and seven univalent *Secale* chromosomes which segregated through the back cross generations. In the case of the *S. vavilovii* amphidiploid, only four F_1 and one BC_1 shrivelled seeds were obtained. Crossing was stopped at this stage due to the sterility of the plants and the growth delay compared to the plants derived from the two other *Secale* species. It is possible that the accession of *S. vavilovii* used was less crossable with wheat and caused a deleterious phenotype.

Wheat/*Secale* introgression lines produced

Genomic *in situ* hybridization techniques were used to characterize the lines produced. A wide range of wheat/*S. anatolicum* and wheat/*S. iranicum* addition and substitution lines were detected. Until now, only the production of introgression lines of wheat/*S. africanum* (Yang et al., 2009) and wheat/*S. montanum* (Miller, 1973, 1984) have been reported. Between one half and five *Secale* chromosomes were detected in the BC_1 lines, while between one half and one complete *Secale* chromosomes were detected in the BC_2 lines. However, no wheat/*Secale* translocation were detected. A high proportion of the *Secale* chromosomes were eliminated through the generations, in 26% of

the BC_1 plants and 72% of the BC_2 plants had lost them. This result of 30% of *Secale* chromosomes retained in the offspring was in agreement with the study on monosomic addition lines (Curtis and Lukaszewski, 1993; Ren et al., 1990). Higher elimination rate in the BC_2 generation was caused by the presence of less *Secale* chromosome in the BC_1 plants compared to the F_1 plants. The highest transmission rate of the *Secale* genome was observed the presence of one complete chromosome, which was transmitted to one third of the offspring. 5% of the offspring contained only a telocentric chromosome, resulting from a centric breakage of the univalent during meiosis (Sears, 1952). In the case of the presence of a telocentric *Secale* chromosome, transmission rate was around 15% only. Szakács and Molnár-Láng, (2010) showed that the transmission rate was dependant on the *Secale* chromosome transmitted in disomic addition lines. Chromosomes 1R and 7R were retained in 90% of the progeny, whereas chromosomes 2R, 3R and 4R were the most unstable. Moreover, most of the lines produced had unbalanced wheat genomes with rearrangements within the wheat genomes (e.g. a small fragment of the A-genome translocated to a D-genome chromosome). One explanation could be the unbalanced genomes of the two initial parents. Most of the *Secale* amphidiploids used had incomplete rye chromosome numbers and also had wheat chromosomes missing. Moreover, chromosome aberrations were increased using the *ph1* mutant as the initial parent. The Paragon *ph1* mutant was composed of 14 chromosomes of each wheat genome, Chinese Spring *ph1* mutant showed some missing or extra chromosomes of the B and D genome. In both of the mutant lines, translocations between wheat genomes were highlighted. In the presence of the *Ph1* locus, i.e. normal Paragon and Chinese Spring wheat, no chromosomic rearrangement was noticed, except the 4AL/7BS intergenomic translocation shared by the allopolyploid wheats (Hao et al., 2013). These results are confirmed by previous studies where it was shown that *ph1* mutant lines had a high level of intergenomic rearrangements; particularly between the A and the D genomes (Sanchez-Moran et al., 2001) or aneuploidy was also noticed. A mean of 0.9 *de novo* rearrangements per plant was found. Genomic rearrangements accumulated through the generations of

multiplication. Sanchez-Moran et al., (2001) proposed to use the *ph2b* mutant (Wall et al., 1971) as no rearrangements were observed in its karyotype. Additional effects need to be considered. For example, the *Ph2* locus is considered as an intermediate (Martinez et al., 2001). Exploitation of the recently produced mutant of the *Tazip4-B2* gene, located in the *Ph1* locus, could be an alternative to the complete *ph1* deletion mutant as it has been shown to be more stable with less accumulation of chromosome translocations (Rey et al., 2017). It has also been demonstrated, that the presence of the rye genome causes chromosome instability, i.e. chromosome aberrations and translocations within the different chromosomes and the different genomes A, B, D and R (Belko et al., 2009; Ren et al., 2017a; Silkova et al., 2017). The fertility of wheat/rye addition lines has been shown to be systematically lower than the wheat cultivar because of the unbalanced genome (Riley, 1960). These chromosome aberrations could explain the reduced self-fertility of the BC_2 lines, especially the ones without any *Secale* chromosomes. In order to reintroduce the *Ph1* locus to stabilize the wheat genomes, normal wheat was used as the recurrent parent. However, by increasing the number of back-cross generations, the number of plants in which the *Secale* genome was totally eliminated increased. Ideally, stabilisation of the *Secale* genome would have been achieved by the introgression of fragments into the wheat genome. But no wheat/*Secale* translocation lines were detected. This could be explained by two possibilities. The first is that despite the deletion of the *Ph1* locus, the very low homology between the *Secale* and the wheat genomes prevented recombination events. At least three translocation events differentiate the wheat genome from the *S. montanum* genome, the ancestor of the genus *Secale* (Devos et al., 1993) and more chromosomal rearrangements are expected in the genome of the different *Secale* species studied. These chromosome rearrangements make a recombination event more difficult. In addition, it has been demonstrated that homologous pairing is preferential over homoeologous paring (Sears, 1977). The second, linked to the first one, is the restriction on the number of plants which can be analysed by the GISH method for each generation. A total of 52

BC_2 plants were germinated with only 15 of them maintaining any *Secale* chromosomes, reducing drastically the probability of finding a wheat/*Secale* translocation event. The use of a higher throughput characterization method, such as molecular markers, would allow a population of a larger size to be screened and thus increase the probability of the detection of translocation lines.

Fertility restoration screening

Back-crossed lines with a single *Secale* chromosome were crossed with CMS lines carrying *T. timopheevii* cytoplasm to score their fertility restoration ability. Only a few of the F_1 resulting from these crosses maintained the *Secale* chromosome (20%). None of the F_1 showed a fertility restoration phenotype. This total sterility could be explained simply by the absence of a restorer factor on the chromosomes present in the F_1. However, a restorer factor could be present but their effect could be masked by the instability of the genomes caused by chromosome aberrations in the different genomes. Results showed that the presence of the *Secale* genome reduced the fertility. Moreover some plants carrying only wheat genomes were not completely fertile because of genome rearrangements.

Conclusion

Addition and substitution lines of wheat/*S. anatolicum* and wheat/*S. iranicum* were successfully developed. Despite the use of *ph1* mutant wheat, no wheat/*Secale* translocation lines were produced. The high level of homoeology between the genomes could be the main explanation. An increase in the size of the population screened could help to detect translocation lines by using high-throughput genotyping methods. In Chapter 4, it was shown that the KASP markers developed by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) were a good high throughput tool to characterize the *S. anatolicum* genome in wheat lines. Chromosome aberrations, such as unbalanced chromosome number or wheat genome rearrangements have been detected and might hide restorer factors against *T. timopheevii*

cytoplasm. In order to completey exploit the potential of *Secale* species to restore *T. timopheevii* cytoplasm other methods to induce translocations into the wheat genome, such as irradiation, might be used.

VI. Chapter 6: Test of wheat/rye introgression lines against *T. timopheevii* cytoplasm

Summary

Wheat/rye introgression lines were tested on CMS lines to localize genes in rye that restore male fertility to hexaploid wheat with *T. timopheevii* cytoplasm. A total of 17 F_1 plants were male fertile with a partial level of restoration. Characterization of the rye chromosomes in these fertile lines was done by cytogenetics methods (GISH) combined to genotyping (KASP markers). Five of the fertile lines did not carry any rye chromosomes which thus suggested that a restorer factor was present in the wheat genome of the male parent, *Rf3* from Chinese Spring. Lines with chromosomes 4RL or 6RL which are known as restorer factors set up 0.65 et 0.40 seeds/spikelet respectively. Highest fertility was observed in the presence of 3RL which is not known to be a restorer chromosome.

VI.1. Introduction

VI.1.1. Secale cereale

Rye (*Secale cereale)*, is a member of the Triticeae tribe. It is a diploid species (2n = 2x = 14, R genome) which originated from Turkey (Salamini et al., 2002). Rye is an allogamous plant in contrast with wheat (Schlegel, 2013). Rye and wheat diverged about 7 million years ago (Marcussen et al., 2014). The rye genome is the largest of the diploid genomes (~7.9 Gbp) (Bartoš et al., 2008) in the Triticeae, with more than 90% repetitive sequences (Flavell et al., 1974). Recently, the first whole genome draft sequence has been published (Bauer et al., 2017). The rye genome has been the subject of numerous structural rearrangements during its evolution (Bauer et al., 2017; Devos et al., 1993; Li et al., 2013; Martis et al., 2013). Synteny relationships between the rye and wheat genomes have been well studied. One of the first RFLP-based genetic maps of rye showed that with the exception of chromosome 1R, all the rye chromosomes have been involved in translocation events (Devos et al., 1993)

(**Figure VI-1**). This synteny map between the rye and wheat genomes has been confirmed with SNP genotyping (Martis et al., 2013). However, some modifications of the synteny map have been proposed after running 79 PCR-based landmark unique gene (PLUG) markers on wheat/rye addition lines (Li et al., 2013). The distal region of the short arm of chromosome 3R is homoeologous with the short arms of wheat group 7 chromosomes and wheat group 6 chromosomes. The distal region of the short arm of chromosome 3R is homoeologous with the short arms of wheat group 3 chromosomes. This study also highlighted the inconsistent position of the centromere of chromosomes 5R, 6R and 7R.

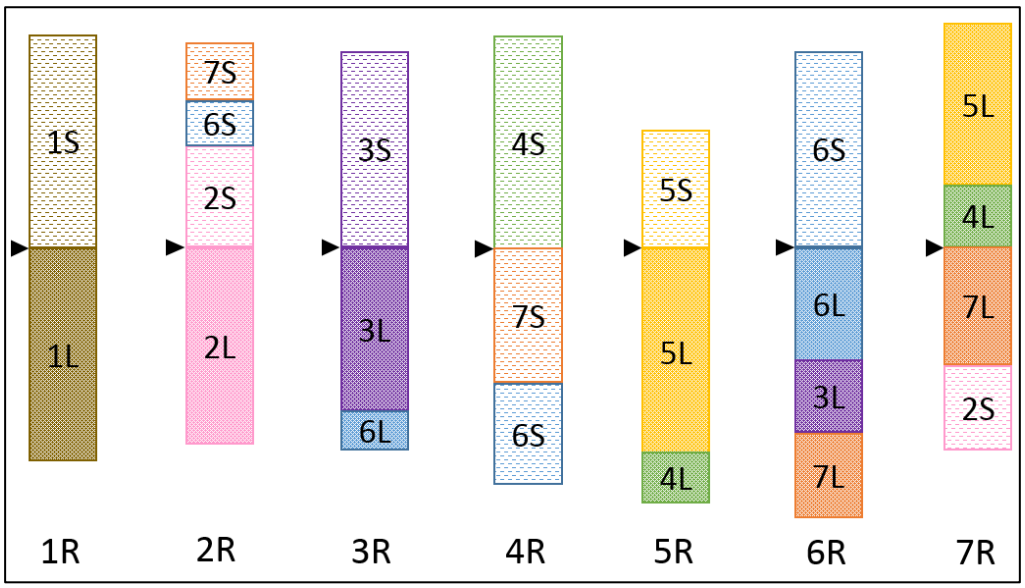


Figure VI-1 Synteny map showing rearrangements of the rye genome compared to the wheat genome (Devos et al., 1993)

The black arrow indicates the centromere

VI.1.2. Rye is a huge reservoir of traits for wheat improvement

VI.1.2.1. *Biotic and abiotic stress resistance genes*

Many studies reported the existence of interesting genes for wheat improvement (**Table VI-1**). Many of the interesting traits shown in **Table VI-1** are located on the short arm of chromosome 1R. In particular, this chromosome confers resistance to leaf rust (*Lr26*), yellow rust (*Yr9*), stem rust (*Sr31*) and powdery mildew (*Pm8*). However, some of these resistance genes, such as *Lr26* (leaf rust), *Pm8* (powdery mildew), *Sr31* (stem rust) and *Yr9* (yellow rust) are not completely effective anymore (Bartos, 1993; Lutz et al.,

1992; Pretorius et al., 2000; Rabinovich, 1998). Chromosome 2R is also an important reservoir of resistance genes, such as for leaf rust (*Lr25* and *Lr45*), stem rust (*Sr59*) or powdery mildew (*Pm7*) resistance. One stem rust resistance gene, *Sr27*, on chromosome 3R and one powdery mildew resistance gene, *Pm20*, on chromosome 6R have also been detected.

Table VI-1 Some interesting traits against biotic and abiotic stress in rye genome

LG	Trait	Locus	Reference
1R	Leaf rust resistance	*Lr26*	(Friebe et al., 1996)
	Powdery mildew resistance	*Pm8*	(Friebe et al., 1996)
	Stripe rust resistance	*Yr9*	(Friebe et al., 1996)
	Stem rust resistance	*Sr31*	(Friebe et al., 1996)
2R	Leaf rust resistance	*Lr25, Lr45*	(Friebe et al., 1996)
	Powdery mildew resistance	*Pm7*	(Friebe et al., 1996)
	Stem rust resistance	*Sr59*	(Rahmatov et al., 2016)
	Hessian fly resistance		(Friebe et al., 1990)
3R	Stem rust resistance	*Sr27*	(Friebe et al., 1996)
	Drought tolerance		(Mohammadi et al., 2003)
	Aluminium tolerance	*Atl2*	(Aniol and Gustafson, 1984)
4R	Stripe rust resistance		(Szakács et al., 2016)
	Frost tolerance		(Erath et al., 2017)
	Aluminium tolerance	*Atl3*	(Aniol and Gustafson, 1984)
	Drought tolerance		(Koeszegi et al., 1996)
5R	Drought tolerance		(Mohammadi et al., 2003)
	Frost tolerance	*Fr-2*	(Erath et al., 2017)
6R	Powdery mildew resistance	*Pm20* *Pm56*	(Friebe et al., 1996) (Hao et al., 2018)
	Aluminium tolerance	*Atl1*	(Aniol and Gustafson, 1984)
	Hessian fly resistance	*H25*	(Friebe et al., 1996)
7R	Drought tolerance		(Mohammadi et al., 2003)
	Frost tolerance		(Erath et al., 2017)
	Aluminium tolerance		(Matos et al., 2005)

VI.1.2.2. *Rye as a donor of restorer genes*

CMS lines with *T. timopheevii* cytoplasm were pollinated by four different sets of CS/rye addition lines (Imperial, King II, Blanco and Dankowskie-Zlote) (Curtis and Lukaszewski, 1993). Some F_1 plants were fertile. Cytological analysis showed that two genes in rye were able to restore the fertility against *T. timopheevii* cytoplasm. The first gene, named as *Rfc3*, was located on the distal region of the long arm of chromosome 6R and completey restored fertility. No allelic variation was found within the four rye cultivars tested. The second gene, named as *Rfc4*, was localized on the long arm of chromosome 4R and caused partial restoration. The level of restoration was dependant on the rye cultivar and also the year. The restoration ability of the rye genome has also been tested in Triticale with *T. timopheevii* cytoplasm and the previous results were confirmed (Góral et al., 2010; Stojalowski et al., 2013).

VI.1.3. Introgression of the rye genome into wheat

The first example of the introgression of the rye genome was the addition of the complete rye genome into wheat which has been illustrated by Triticale (*Triticosecale* Wiitmack), a man-made hybrid created in 1875 by a Scottish scientist A. Stephen Wilson. This new hybrid combined the agronomic performance and quality of wheat with the stress tolerance of rye (Mergoum et al., 2009; Randhawa et al., 2015). Many rye fragments have been successfully transferred into the wheat genome (**Table VI-2**). The translocations occurred spontaneously (centric breakage fusion) (Friebe et al., 1996), induced by irradiation (Ahmad et al., 2000; Friebe et al., 1996) or through the use of the *ph1* mutant (Miller et al., 1994; Naranjo and Fernández-Rueda, 1996). The gametocidal chromosome $4M^g$ of *Ae. geniculata* has also been used to successfully induce translocations between the wheat and rye genomes in Triticale after fragmentation of the chromosomes (Kwiatek et al., 2017).

Table VI-2 List of some spontaneous and induced wheat/rye translocations

Translocation	Method used	Reference
1RS.1BL	Centric breakage-fusion	(Lein, 1975)
1RS.1AL	Irradiation	(An et al., 2013)
1RS.1DL	Centric breakage-fusion	(Lukaszewski, 1997)
2RS.2BL	Centric breakage-fusion	(Graybosch, 2001)
2BS.2RL	Centric breakage-fusion	(Graybosch, 2001)
2RL.2BL	*ph1* mutant	(Naranjo and Fernández-Rueda, 1996)
3AL.3RS	Centric breakage-fusion	(Marais, 2001)
3BL.3RS	Centric breakage-fusion	(Marais, 2001)
4BL.4RS	Centric breakage-fusion	(An et al., 2013)
4BL.4RS	Gametocidal gene	(Kwiatek et al., 2017)
7AS.4RS	Centric breakage-fusion	(An et al., 2013)
5DS-4RS.4RL	Irradiation	(Fu et al., 2014b).
4RS-5DS.5DL	Irradiation	(Fu et al., 2014b).
5BS.5RL	Centric breakage-fusion	(Merker, 1992)
5BS-5BL.5RL	Gametocidal gene	(Kwiatek et al., 2017)
6RS.6AL	Centric breakage-fusion	(Hao et al., 2018)
6BS.6BL-6RL	Irradiation	(Friebe et al., 1991)
4BS.4BL-6RL	Irradiation	(Friebe et al., 1991)
4AS.4AL-6RL-4RL	Irradiation	(Friebe et al., 1991)
4A.7R	Centric breakage-fusion	(Zeller and Koller, 1981)
7RS.3AL	Gametocidal gene	(Kwiatek et al., 2017)

The translocation 1RS.1BL is the most famous. This translocation occurred in the hybrid between wheat and the rye cultivar “Pektus” in 1924 during the interspecific crossing programme of Georg Riebesel (Lein, 1975). The first commercial cultivar containing this translocation was released in 1957 and now this 1RS.1BL translocation is widespread in hundreds of wheat cultivars (Lukaszewski, 1990; Rabinovich, 1998). Success of this translocation is mainly due to the presence of many disease resistance gene on 1RS (Crespo-Herrera et al., 2017; Friebe et al., 1996). However, 1RS is known to have deleterious

effects on bread quality such as sticky dough, poor fermentation, inferior dough-mixing properties, and low SDS sedimentation volumes (Burnett et al., 1995; Graybosch, 2001). This is caused by the presence of *Sec-1*, a complex locus conditioning the production of several rye secalin proteins (Dhaliwal and MacRitchie, 1990; Hussain and Lukow, 1994). Strategies of chromosome engineering by induced homoeologous recombination have been used to remove the associated bread making quality defect (Lukaszewski, 2001). New sets of disomic 1RS.1BL translocation lines, which carry the 1RS.1BL chromosome with partial or whole gene deletions at the *Sec-1* locus, have been produced and these lines harbour better bread qualities (Li et al., 2016b). Chromosome 1RS has also been transferred to wheat in the form of a 1RS.1AL translocation via X-ray treatment (An et al., 2013). This translocation line was produced in the 1970s and is mainly used in the USA (Jung and Seo, 2014; Lukaszewski, 1990). 1RS.1AL lines have fewer deleterious effects on bread making quality than the 1RS.1BL lines (Graybosch, 2001; Graybosch et al., 1993; Kim et al., 2005). Translocations involving the other rye chromosomes have been developed but are less widely used (**Table VI-2**).

One common characteristic of most of these wheat/rye translocation lines is the insertion of a rye genome fragment of a large size, most of the time a complete arm. The ultimate aim of an introgression is to reduce the size of the rye segment in the wheat genome as much as possible to avoid linkage drag. Until now this has only been achieved by radiation treatment. For example, the translocation line 4BS.4BL-6RL contains only the distal half of 6RL and a very small segment (about 1 µm) of the distal region of chromosome 6RL inserted in the long arm of chromosome 4A (Friebe et al., 1991).

VI.1.4. Characterization of the wheat/rye introgression lines

Identification of the wheat/rye translocations lines is an essential part of an introgression programme to speed up the selection process and to precisely localize the interesting traits. For that purpose, a large number of molecular markers such as RFLP, AFLP, SSR markers have been developed (Kofler et al.,

2008; Miftahudin et al., 2002; Rogowsky et al., 1993). More recently an Axiom® Wheat/Wild relative 35K SNP array has been developed with SNPs showing polymorphism for ten wild relatives (including rye) to wheat (Winfield et al., 2016). These SNP markers were converted in KASP markers by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham). These markers became available in summer 2018. Cytogenetic techniques, i.e. GISH and FISH, have been successfully developed as well, to detect the presence of the rye genome in a wheat background. Two FISH probes are usually used as they allow non ambiguous differentiation and identification of the different chromosomes: the probe pSc119.2 (Bedbrook et al., 1980) identifies the rye genome, the B-genome and some A and D-genome chromosomes and the probe pAs1 (Bedbrook et al., 1980) identifies the A and D- genome chromosomes (An et al., 2015; Ren et al., 2017b; Wang et al., 2009).

VI.1.5. Aim and objectives of the chapter

The aim of this chapter was to study the rye genome for its potential to restore *T. timopheevii* cytoplasm

- To test wheat/rye introgression lines produced at the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) on CMS lines
- The identification of the fertile lines by cytogenetic and molecular techniques to ultimately detect a line with a small fragment of rye restoring completey *T. timopheevii* cytoplasm.

VI.2. Material and methods

VI.2.1. Plant material

Four different hexaploid wheat varieties carrying *T. timopheevii* cytoplasm were used as the female parent. Two lines were spring type and two lines were winter type. The CMS lines were provided by Limagrain and were at the BC_6 generation. Wheat/rye introgression lines were used as pollinators. A large panel of wheat/rye introgression lines were developed by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham). The

initial cross consisted in pollination of Chinese Spring *ph1* mutant by two different accessions of *Secale cereale* (PI 390382 and PI 428373). A back cross programme was carried out by using common wheat as the recurrent male parent (Chinese Spring or Paragon). The complete pedigree of the lines used is shown in Appendix 2. In collaboration with the University of Bristol, an Axiom® Wheat/Wild relative 35K SNP array has been developed with SNPs showing polymorphism for ten wild relatives (including rye) to wheat (Winfield et al., 2016; King et al., 2017). Based on the genotyping data of their parental lines, 57 different introgression lines were selected for the potential presence of chromosome 4R or 6R.

VI.2.2. Crossing programme

Figure VI-2 shows the crossing scheme. For the production of the F_1 generation, CMS wheat was pollinated by wheat/rye introgression lines. As the CMS lines were used as female, there was no need for emasculation. Each head of the CMS line was covered with a bag before the opening of the spikelets to avoid pollen contamination (refer to Chapter II.1.4). Once the stigmas were fluffy and receptive, heads were pollinated with wheat/rye introgression line pollen. After pollination, heads were bagged to protect them.

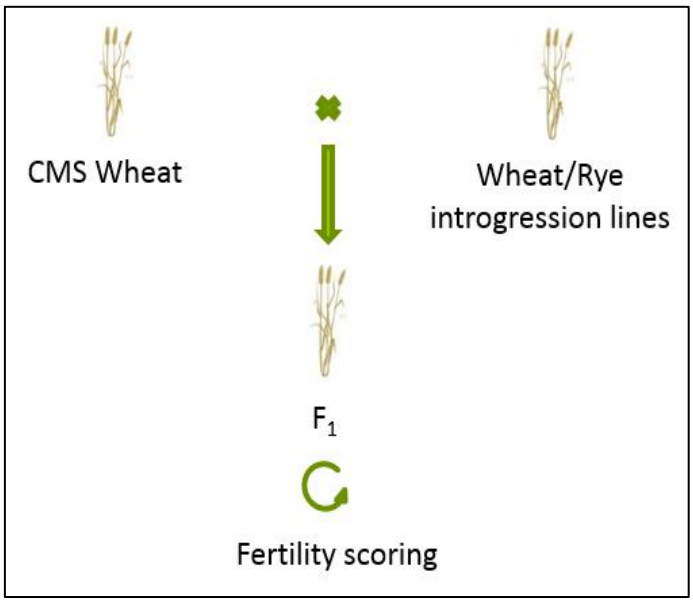


Figure VI-2 Crossing scheme to screen the ability of wheat/rye introgression lines to restore *T. timopheevii* cytoplasm

VI.2.3. Seed germination

Germination of the seeds was carried out according to the protocol (refer to Chapter II.1.1). Once the seed germinated, it was sown into soil (John Innes compost No. 2). Plants were put for four weeks into vernalisation (at 8°C with 12 hours light per day) and then transferred to the glasshouse (at 21°C, 16 hours light per day).

VI.2.4. Fertility restoration scoring

To score the male fertility restoration, three heads of the F_1 were bagged prior to anthesis and harvested after ripening. For each selfed spike, the number of seeds as well as the number of spikelets were counted and both were averaged by plant. The seed set of a plant was defined as the number of seeds divided by the number of spikelets. A plant without any selfed seed was considered as male sterile, whereas a plant with at least one seed was considered as male fertile. Common wheat was used as a control.

VI.2.5. Cytogenetic analysis

Preparation of metaphase spreads of the fertile lines was described in Chapter II.3.3. Slides were initially probed using total genomic DNA of *Ae. speltoides* as blocking DNA and total genomic DNA of rye labelled by nick translation with Alexa Fluor 546-14-dUTP (Invitrogen; C11401) (gold), total genomic DNA of *T. urartu* labelled by nick translation with Alexa Fluor 488-5-dUTP (Invitrogen; C11397) (green) and total genomic DNA of *Ae. tauschii* labelled with Alexa Fluor 594-5-dUTP (Invitrogen; C11400) (red).

VI.2.6. KASP markers

A total of 1000 KASP markers, polymorphic between four wheat cultivars (Chinese Spring, Highbury, Paragon and Pavon 76) and ten wild relatives (including *S. cereale*) have been developed by the Nottingham/BBSRC Wheat Research Centre in collaboration with LGC. These 1000 KASP markers have been validated on 5000 genotypes, including the wild accessions of rye and the wheat parents. Out of the markers validated, those polymorphic between the

different wheats and the wild rye accessions were selected, leading to a set of 188 markers. 143 markers were assigned to one of the rye linkage group according to the previous genotyping data done at the Nottingham/BBSRC Wheat Research Centre (University of Nottingham). From these 146 markers, a total of 103 markers were selected to cover the seven linkage groups of the rye genome. Genomic DNA extraction of the CS/rye addition lines (1R to 7R) (Miller, 1973) and the developed fertile lines was done by the method explained in Chapter II.2 PCRs were run by the protocol explained in Chapter 2.VI.2. Each mixture was composed of 5µL of DNA, 2.43 µL of dH_2O, 2.50 µL of ROX PACE mix and 0.068 µL of primers mix. The details of the PCR cycle were given in Chapter II.4.2.

VI.3. Results

VI.3.1. F_1 generation production

A total of 157 CMS heads were pollinated producing 716 F_1 seeds. However, crosses done with eleven introgression lines did not set any seeds, mainly because of the poor pollen production of these lines. A selection of F_1 seed were germinated in order to cover all the combinations of CMS x wheat/rye introgression lines. A total of 251 F_1 were sown, of which 213 F_1 germinated and reached the adult stage.

VI.3.2. Fertility restoration scoring

A total of 17 F_1 plants set selfed seeds and thus these plants were considered as fertile whereas the other 196 F_1 plants were sterile. Eleven F_1 set less than 0.5 seed/spikelet, four plants between 0.5 and 1 seed/spikelet and two plants set more than 1 seed/spikelet with a maximum of 1.54 seeds/spikelet (**Figure VI-3** and **Figure VI-4**). The wheat control set 2.4 seeds/spikelet. A total of 633 F_2 seeds were produced.

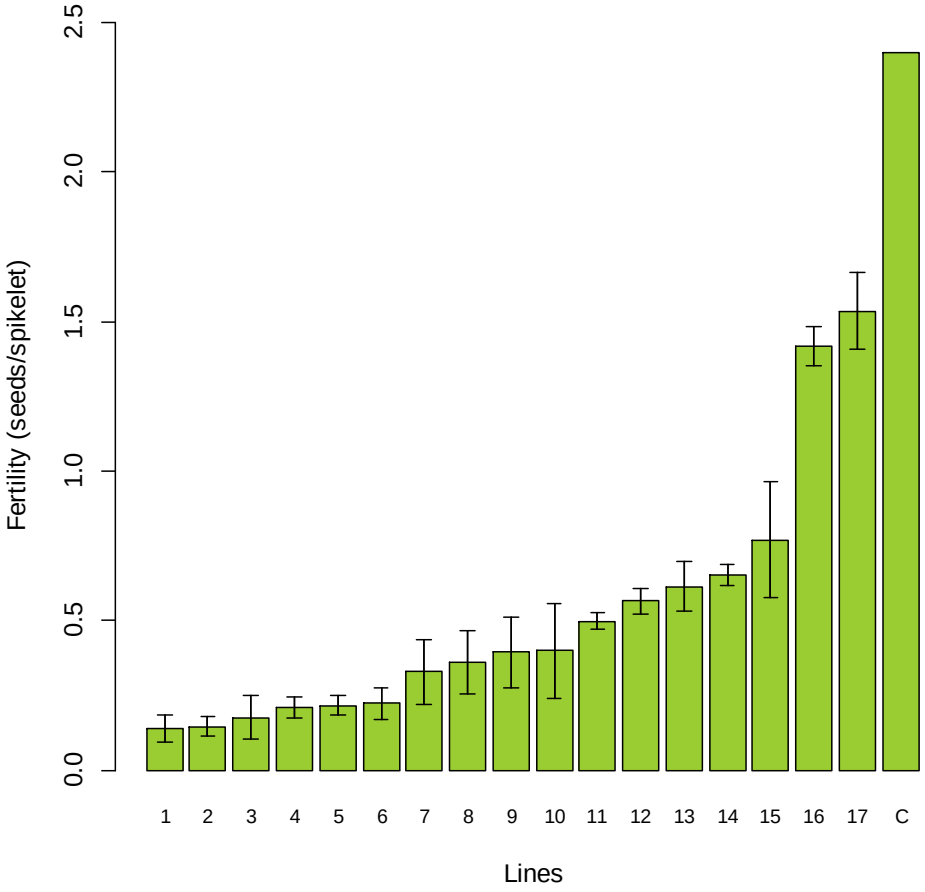


Figure VI-3 Number of seeds/spikelet set by the fertile F_1 plants

Genotype numbers 1 to 17 are the F_1 lines, C designated the control (wheat)

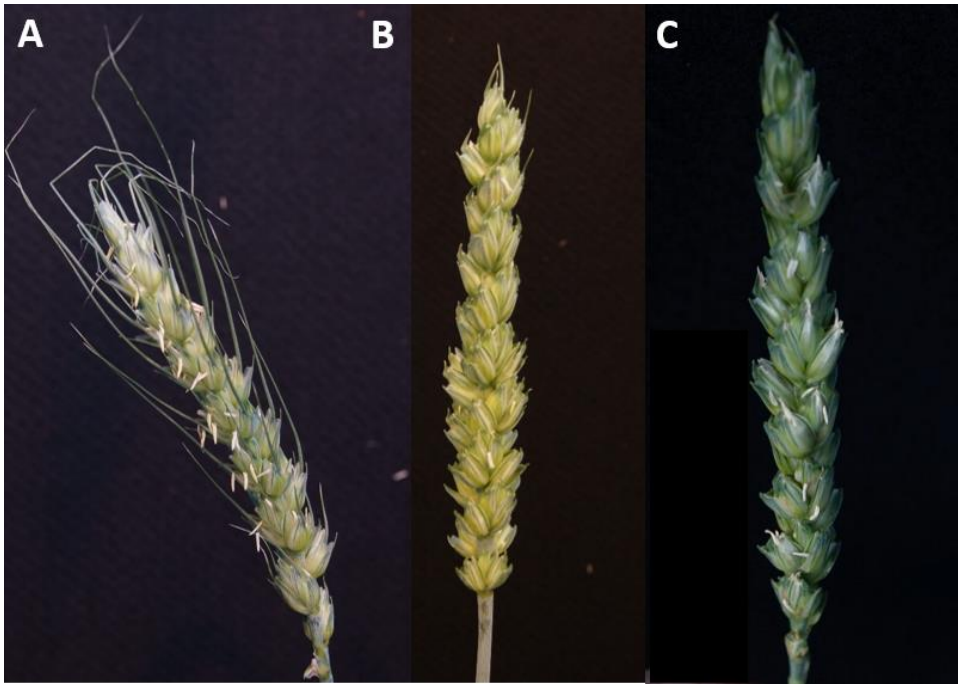


Figure VI-4 Head shapes of fertile lines

A) F_1.9 (0.39 seeds/spikelet), B) F_1.10 (0.40 seeds/spikelet) and C) F_1.13 (0.61 seeds/spikelet)

VI.3.3. Characterization by Genomic *in situ* Hybridization

Genomic composition of the fertile lines was determined by using multi-colour GISH to distinguish the chromosomes of the A, B, D and R genomes (**Figure VI-5** and **Table VI-3**). The metaphase spread quality of lines 3, 8, 12 and 13 was of insufficient quality to distinguish the chromosomes of the three wheat genomes. Five F_1 did not have any rye chromosome (lines 1, 5, 6, 12 and 13) (**A**), seven carried a telocentric rye chromosome (lines 3, 7, 8, 11, 15, 16 and 17), four F_1 had a complete rye chromosome (lines 2, 9, 10 and 14) and one F_1 had a complete and one telocentric rye chromosome (line 4) (**B**). Multi-colour GISH revealed unbalanced wheat chromosome numbers. For example F_1.4 was composed of 13 A-genome chromosomes, 17 B-genome chromosomes and 14 D-genome chromosomes (**B**) and F_1.5 was composed of 15 A-genome chromosomes, 13 B-genome chromosomes and 14 D-genome chromosomes (**A**) intergenomic translocations were observed as well (**B**).

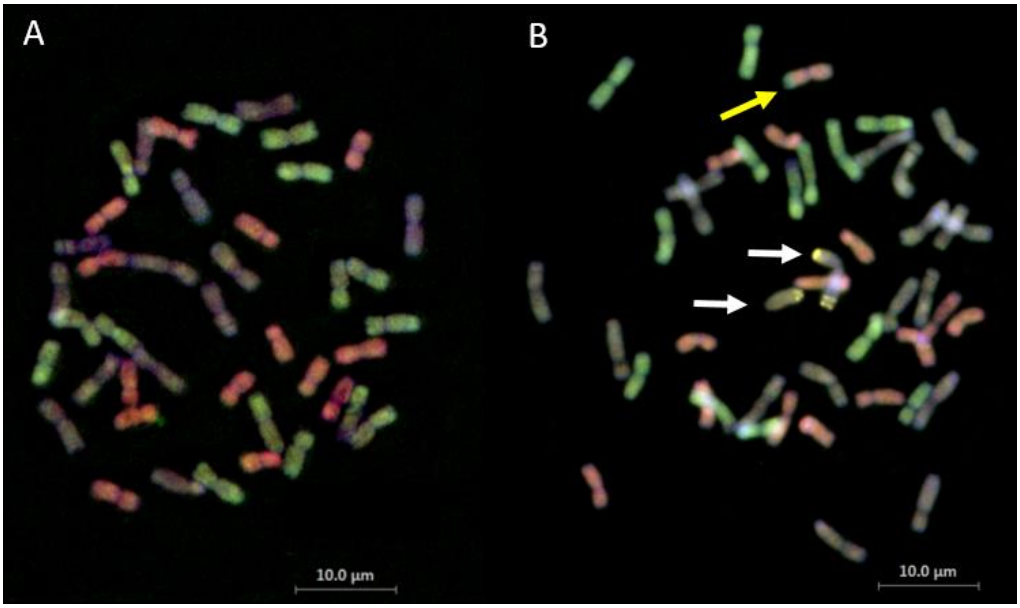


Figure VI-5 Multi-colour GISH images of two F_1 fertile lines

A genome in green, B genome in blue, D genome in red and R genome in gold indicated by the white arrows. (A) F_1.5 (15A + 13B + 14D) and (B) F_1.4 (13 A + 17B + 14D + 1.5R). Yellow arrow indicates a translocation within the wheat genomes.

Table VI-3 Genomic composition of the F_1 fertile lines

F_1 code	R genome	A genome	B genome	D genome
1	0	14	14	14
2	1	14	13	14
3	0.5	-	-	-
4	1.5	13	17	14
5	0	15	13	14
6	0	14	13	14
7	0.5	15	13	14
8	0.5	-	-	-
9	1	14	14	14
10	1	14	14	14
11	0.5	15	13	14
12	0	-	-	-
13	0	-	-	-
14	1	14	15	14
15	0.5	14	14	14
16	0.5	13	13	15
17	0.5	14	14	15

VI.3.4. Characterization by genotyping

Out of the 146 KASP markers available, a total of 103 markers were run on the most fertile F_1 lines with 66 markers polymorphic between the wheat parents and rye (**Table VI-4**).

Table VI-4 Number of KASP markers available, tested and confirmed for each linkage group of rye

Linkage group	No. of polymorphic markers	No. of marker tested	No. of markers working
1	27	16	9
2	22	16	9
3	18	13	11
4	18	18	9
5	22	12	9
6	25	16	13
7	14	12	6
Total	146	103	66

The positions of the markers were confirmed by the addition line results. For this reason, two markers were repositioned, one from chromosome 2R to 6R and one from chromosome 7R to 4R (**Figure VI-6**). Chromosomes 1R, 1RL, 1RS, 4R, 4RL, 6RS, 6RL and 7RL were detected in the different fertile lines.

		Addition lines							Fertile lines											
LG	Marker	1R	2R	3R	4R	5R	6R	7R	2	3	4	7	8	9	10	11	14	15	16	17
	WRC0005	H	W	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	W
	WRC0059	H	W	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	W
	WRC0057	W	W	W	W	W	W	W	H	W	W	W	W	?	W	W	W	W	H	W
	WRC0074	R	W	W	R	W	W	W	R	W	R	W	W	W	W	R	W	W	H	W
1R	WRC0140	H	W	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	H	W
	WRC0034	H	W	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	H	W
	WRC0037	H	W	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	H	W
	WRC0091	W	W	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	H	W
	WRC0100	H	W	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	W
	WRC0254	W	H	W	W	W	W	W	W	W	W	W	W	W	W	H	W	H	W	W
	WRC0152	W	H	W	W	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W
	WRC0155	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0159	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0208	W	H	?	H	?	?	?	W	W	W	W	W	W	W	?	W	W	?	W
2R	WRC0218	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0281	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0184	W	H	W	W	W	W	W	R	W	R	W	W	R	R	W	R	W	R	W
	WRC0192	W	H	W	W	W	W	W	W	W	W	W	W	H	W	W	W	W	W	W
	WRC0238	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0239	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0300	W	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0391	W	W	H	W	W	W	W	W	W	W	H	W	W	W	H	W	W	W	W
	WRC0395	W	W	H	W	W	W	W	W	W	W	H	W	W	W	H	W	W	W	H
	WRC0400	W	W	H	W	W	W	W	W	W	W	H	W	W	W	H	W	H	W	H
	WRC0304	W	W	H	W	W	W	W	W	W	W	H	W	W	W	H	W	W	W	H
3R	WRC0352	W	W	H	W	W	W	W	W	W	W	H	W	W	W	R	W	W	W	H
	WRC0403	W	W	H	W	W	W	W	W	W	W	H	W	W	W	H	W	W	W	H
	WRC0418	?	W	H	?	W	?	R	?	?	?	W	W	?	?	W	?	W	W	?
	WRC0421	W	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0370	W	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0373	W	W	H	W	W	W	W	W	W	W	W	W	?	W	W	W	W	W	W
	WRC0330	W	W	H	W	W	W	W	W	W	W	W	W	H	W	W	W	W	W	W
	WRC0461	W	H	W	H	W	W	W	W	H	H	W	H	W	W	W	W	H	H	W
	WRC0460	W	W	W	H	W	W	W	W	W	H	W	W	W	W	W	H	W	W	W
	WRC0468	W	W	W	H	W	W	W	W	W	H	W	W	W	H	W	H	W	W	W
	WRC0522	W	W	W	H	W	W	W	W	W	H	W	W	W	H	W	H	W	W	W
4R	WRC0536	W	W	W	H	W	W	W	W	H	H	W	W	H	W	W	H	W	W	W
	WRC0908	W	W	W	H	W	W	W	W	H	H	W	W	H	W	W	H	W	W	W
	WRC0866	W	W	W	H	W	W	W	W	H	H	W	?	H	W	W	H	W	W	?
	WRC0911	W	W	W	H	W	W	W	W	H	H	W	W	H	W	W	H	W	W	W
	WRC0811	W	W	W	H	W	W	W	W	H	H	W	W	R	W	W	H	W	W	W
	WRC0518	W	W	W	W	H	W	W	W	W	W	W	W	W	W	H	W	W	W	W
	WRC0614	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0675	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0570	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W
5R	WRC0571	W	W	W	W	W	W	W	W	W	W	W	H	W	W	W	W	W	W	W
	WRC0576	W	W	W	W	H	W	W	W	W	W	W	H	W	W	W	W	W	W	W
	WRC0628	W	W	W	W	H	W	W	W	W	W	W	H	W	W	W	W	W	W	W
	WRC0692	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0639	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0818	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	H	W	W
	WRC0768	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	H	W	W
	WRC0828	W	W	W	W	W	H	W	H	H	W	W	W	?	H	W	W	H	H	W
	WRC0777	?	R	?	W	?	H	?	?	W	W	W	?	?	?	W	W	W	W	W
	WRC0733	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	H	W	W
	WRC0737	W	W	W	W	W	H	W	W	W	W	W	W	W	H	W	W	W	W	W
	WRC0850	W	W	W	W	W	H	W	W	W	W	W	W	W	H	W	W	H	W	W
6R	WRC0851	W	W	W	W	W	H	W	W	W	W	W	W	W	H	W	W	W	W	W
	WRC0749	W	W	W	W	W	H	W	W	W	W	W	W	W	H	W	W	W	W	W
	WRC0750	W	W	W	W	W	H	W	W	W	W	W	W	W	H	W	W	W	W	W
	WRC0751	W	W	W	W	W	H	W	W	W	W	W	W	W	H	W	W	H	W	W
	WRC0802	W	W	W	W	W	W	W	W	W	W	W	W	W	H	W	W	W	W	W
	WRC0336	W	W	W	W	W	H	W	W	W	W	W	W	W	H	W	W	W	W	W
	WRC0998	W	W	W	W	W	H	W	W	W	W	W	W	W	H	W	W	W	W	W
	WRC0544	W	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	H	W	W
	WRC0439	W	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	W	W
7R	WRC0436	W	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	H	W
	WRC0931	W	W	W	W	W	W	H	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0992	W	W	W	W	W	W	H	W	W	H	W	W	W	W	W	W	W	W	W
	WRC0984	W	W	W	W	W	W	H	W	W	H	W	W	W	W	W	W	W	W	W

Figure VI-6 Graphical representation of the KASP genotyping of the CS/*Secale* addition lines and the fertile lines produced

Markers are ordered by linkage groups and are scored as “W” for wheat allele, “H” for heterozygous and “S” for Secale allele. Missing values are indicating by “?”

VI.3.5. Comparison of GISH and KASP results

Table VI-5 summarizes the results obtained by GISH and KASP marker analysis of the fertile F_1 lines. The results were consistent for lines 2, 3, 4, 7, 8, 11, 14, 15, 16 and 17.

Table VI-5 Combination of the GISH and KASP rye genome characterization of the fertile lines

F_1 code	Chromosome number (GISH)	Chromosome (KASP)	Fertility
2	1	1R	0.15
3	0.5	4RL	0.18
4	1.5	4R + 7RL	0.21
7	0.5	3RS	0.33
8	0.5	5RL	0.36
9	1	4RL	0.39
10	1	4RS + 6RL	0.4
11	0.5	3RS	0.5
14	1	4R	0.65
15	0.5	6RS	0.77
16	0.5	1RL	1.42
17	0.5	3RL	1.54

For example, one complete chromosome of rye was detected on the metaphase spread of line 2 (**Figure VI-7**). For this line, KASP markers heterozygous between rye and wheat genome were all located on chromosome 1R. Thus it was concluded that line 2 had a complete 1R chromosome. However, for lines 4, 9 and 10, a combination of the GISH and KASP markers results was needed to correctly identify the rye chromosome. GISH analysis allowed the detection of one complete rye chromosome on line 9 whereas genotyping indicated the presence of the long arm of chromosome 4R only (**Figure VI-7**). Line 9 actually carried two long arms of chromosome 4 which fused to form a single chromosome. The same scenario could be transposed to line 10 in which one complete rye chromosome was detected

by GISH whereas the genotyping indicated the presence of the small arm of chromosome 4R and the long arm of chromosome 6R (**Figure VI-7**). Thus the rye chromosome present in line 10 seemed to be a rye/rye translocated chromosome 4RS.6RL.

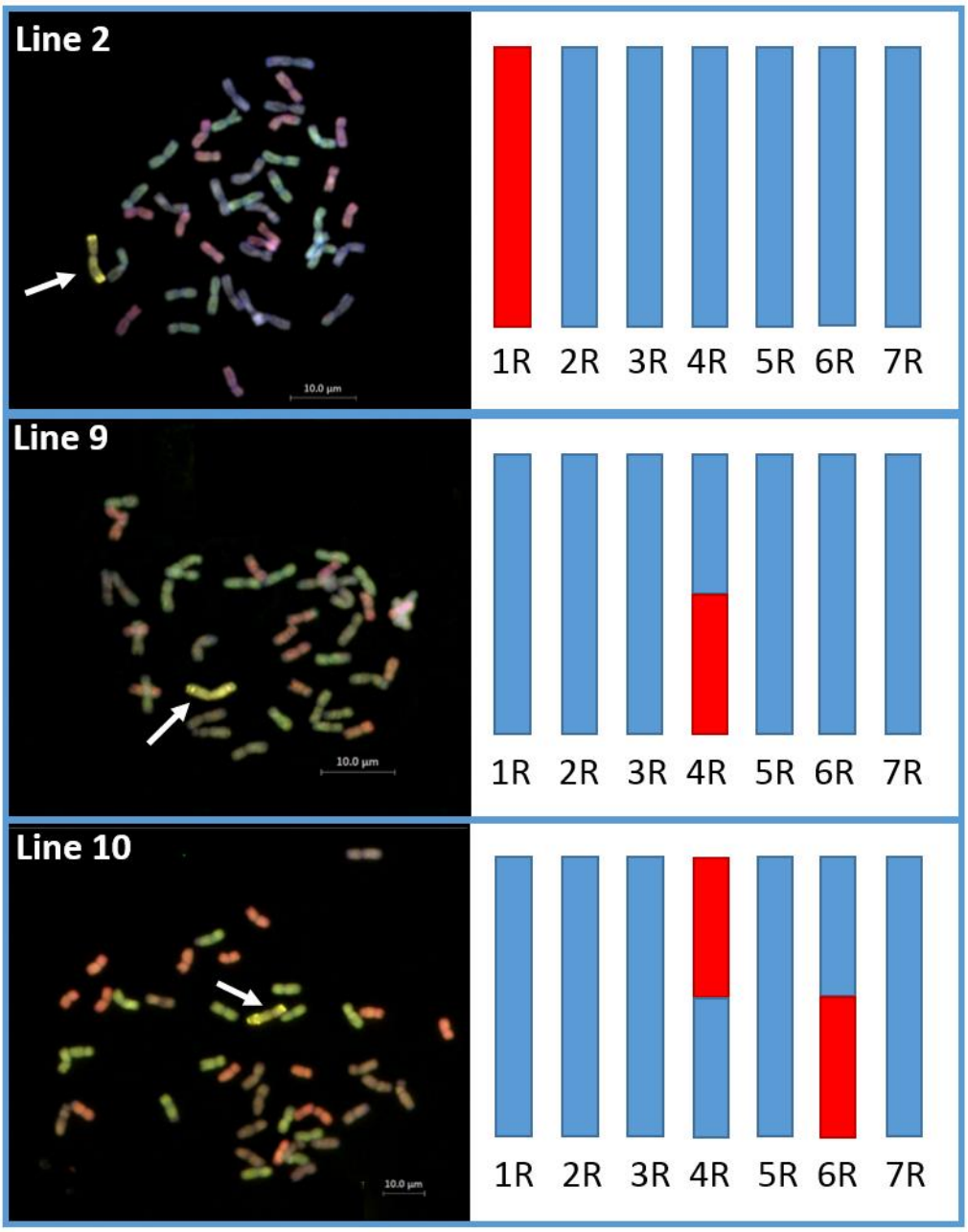


Figure VI-7 Comparison between GISH and KASP marker results of some fertile lines

On the left, multi-colour GISH image (A genome in green, B genome in blue, D genome in red and R genome in gold indicated by the white arrows). On the right genotyping results, blue colour indicates the wheat allele and red colour the rye allele

The two lines with the highest fertility level, F_1.16 and F_1.17, contained rye chromosomes 1RL and 3RS respectively with 1.42 and 1.54 seeds/spikelet respectively. The presence of chromosome 4RL was linked to a fertility ranging from 0.18 to 0.65 seeds/spikelet and a F_1 with chromosome 6RL set 0.4 seed/spikelet.

VI.4. Discussion

Several genes have been identified in the wheat genome as restorers of fertility against *T. timopheevii* cytoplasm (Bahl and Maan, 1973; Tahir and Tsunewaki, 1969; Yen et al., 1969). However, several restorer genes have to be combined to completely restore the fertility (Sage, 1972; Wilson, 1968b). In addition to the major *Rf* genes, minor loci, named as modifiers, are needed to ensure a stable and complete restoration (Maan et al., 1984; Robertson and Curtis, 1967). However, it has been shown that the addition of the long arm of chromosome 6R completely restored *T. timopheevii* cytoplasm, whereas addition of the long arm of chromosome 4R caused partial restoration (Curtis and Lukaszewski, 1993). The rye genome therefore appeared as an interesting source of restorer genes for hybrid wheat based on CMS- *T. timopheevii*. In addition, chromosome 4R carries two genetic factors associated with increased pollen grain number and anther size, on the short and long arm respectively, which are two desirable traits for the parental male in a CMS system (Nguyen et al., 2015).

Two hypotheses were looked at in preliminary work to this chapter. The first one was the presence of a complete chromosome arm opposed to a small fragment of rye could hide potential restorer factors by inducing sterility because of linkage drag and the unbalanced genome. The second hypothesis was that in rye germplasm different alleles of the *Rf* genes are already known and thus new *Rf* genes might be present.

A lot of work has been undertaken to introgress interesting traits such as disease resistance from rye into the wheat genome, but very few initial donors

were used and some case of resistance breakdown have been reported (Crespo-Herrera et al., 2017). In order to capture more diversity, the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) started a crossing programme with different wild rye accessions (PI 390382 and PI 428373). Several rearrangements have occurred during the evolution of the wheat and rye genomes, and thus recombination events between the genomes of these two species have occurred at low frequency (Devos et al., 1993). In order to increase the probability of recombination between the rye and wheat genomes, *ph1* mutant Chinese Spring was used as the initial female line (Sears, 1977). Chinese Spring also has the recessive alleles *kr1* and *kr2* showing higher crossability with rye (Riley and Chapman, 1967). Back-crosses were carried out using an elite cultivar, Paragon (Angus et al., 2011). This material has been characterized by the Axiom® Wheat/Wild relative 35K SNPs array. A panel of these wheat/rye introgression lines was selected to carry either 4R or 6R as well as other rye linkage groups and used as the pollen donors on CMS-lines with *T. timopheevii* cytoplasm. The fertility of a total of 213 F_1 plants was scored. Seventeen F_1s set self-seeds ranging from 0.15 to 1.54 seeds/spikelet, compared to selfed common wheat which set an average of 2.4 seeds/spikelet.

Many studies have reported the low transmission rate of wheat/wild relative introgression through the generations (Ren et al., 1990; Whelan et al., 1986). Thus, the fact that less than 10% of the off-spring set self-seeds could be explained by the relatively high loss rate of the rye chromosomes (and thus the *Rf* factor) through the generations. Genotyping by the Axiom® Wheat/Wild relative 35K SNP array had been carried out on the parental lines of the pollen donors of the F_1 which are thus separated by two meiosis events. Two meiosis events occurred between these genotyped lines and the F_1 generation causing rye chromosome segregation.

Fertile F_1 lines were characterized by multi-colour GISH. Five of the fertile lines did not carry any rye chromosomes which thus suggested that a restorer factor

was present in the wheat genome of the male parent. The fertility of these lines ranged from 0.14 to 0.61 seeds/spikelet. Two wheat genotypes were used in the pedigree of the wheat/rye introgression lines, Paragon and Chinese Spring. It has been shown in the Chapter 3 that Paragon was not able to restore *T. timopheevii* cytoplasm as the F_1s between the CMS lines and Paragon were all sterile. On the contrary, in the same chapter, fertility of the F_1 between some CMS genotypes and Chinese Spring was reported leading to the conclusion that Chinese Spring carried a *Rf* gene against *T. timopheevii* cytoplasm. Monosomic analysis led to the conclusion that chromosome 1B of CS was carrying a dominant restoring gene causing partial fertility restoration, which could be *Rf3* (Kučera, 1982). However, Chinese Spring was used as the initial parent in the original crossing scheme followed by several back-crosses with Paragon. Chromosome 1B of CS might thus have been eliminated or be present in a monosomic condition in the wheat/rye lines used as male parent explaining the low proportion of fertile plants observed. Another hypothesis could be the occurrence of a spontaneous 1RS.1BL translocation, which caused the loss of 1BS. This 1RS.1BL translocation line has already been studied as a potential sterile line in CMS-systems based on the cytoplasms of *Ae. kotschyi*, *Ae. mutica* and *Ae. uniaristata* (Tsunewaki, 2015). This chromosome has been engineered to reduce the size of the rye chromosome in order to reduce the linkage drag (Lukaszewski, 2017). However, *Rf3* could be present in the sterile lines but its expression prevented by the presence of other rye chromosomes, the absence of enhancer loci or the presence of inhibitor loci in either the wheat or rye genomes (Bahl and Maan, 1973; Sage, 1972; Wilson, 1968b).

The twelve fertile F_1s carried from a telocentric to a complete and a half rye chromosome. No wheat/rye translocated chromosomes were found. It has been shown that where a translocated chromosome is present, less that 10% of the offspring contained the translocated chromosome when it was transmitted through the pollen (Whelan et al., 1986). Most of the F_1 plants presented an unbalanced wheat genome, e.g. one chromosome of one genome was missing and 15 chromosomes of another genome were present.

The presence of wheat intergenomic translocations were also observed. Several chromosomal rearrangements and aneuploidy were found in the *ph1* mutant wheats used as initial parents (refer to Chapter V.3.2.2). An unbalanced genome might also be the result of an abnormal meiosis because of the presence of rye univalent chromosomes.

The fertile lines carrying rye chromosomes were genotyped with 66 KASP markers developed by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) to characterize the rye genome. F_1 plants carrying chromosome 4RL set 0.18, 0.21, 0.39 and 0.65 seeds/spikelet, a fertility restoration level lower than the one reported by Curtis and Lukaszewski, (1993), (from 0.57 to 1.65 seeds/spikelet). F_1s carrying chromosome 6RL set 0.4 seeds/spikelet, again a fertility restoration level lower than the one reported by Curtis and Lukaszewski, (1993), (from 1.94 to 2.60 seeds/spikelet). The presence of a less efficient allele of the restorer gene, an unbalanced genome or chromosome aberration caused by the presence of the rye genome (Belko et al., 2009; Lukaszewski and Gustafson, 1983; Ren et al., 2017a; Silkova et al., 2017) could explain the lower seed set. Seven of the fertile F_1 plants carried rye chromosomes which were not known to have restorer genes. The F_1s with the highest fertilities carried the long arm of chromosome 1R (1.42 seeds/spikelet) and the short arm of chromosome 3R (1.54 seeds/spikelet). The fertility level of these F_1s was similar to the F_1s between the CMS lines and Chinese Spring (up to 1.23 seeds/spikelet). Hence, it is possible that the fertility restoration observed in these lines was due to the presence of the restorer chromosome of Chinese Spring and not the rye chromosomes.

<u>Conclusion</u>

Previous studies have shown that rye is a good source of *Rf* genes to restore *T. timopheevii* cytoplasm in a wheat background. In order to avoid the fertility penalties caused by linkage drag and unstable genomes, wheat/rye introgression lines produced by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) were tested on the CMS-line. Very few F_1

lines were found to be fertile. Characterization of these lines was carried out by cytogenetic and molecular marker analysis. No wheat/rye translocation lines were detected. Some of the fertile lines did not carry any rye chromosomes, other lines carried rye chromosomes which were not known to carry restorer genes and the lines with known rye restorer chromosomes showed a much lower fertility level than expected. However, the number of plants was too low to confirm the presence of a restorer gene on the Chinese Spring accession used in the programme. More back-crossed generations with non-restorer wheats are needed. The newly developed KASP markers have been successfully confirmed by GISH methods. These markers could be used to characterize the lines used as pollen donors in order to increase the efficiency of the crossing. Moreover, a larger F_1 population could be screened, thus increasing the chance of finding wheat/rye translocated chromosome to restore *T. timopheevii* cytoplasm.

VII. Chapter 7: Test of wheat/*T. timopheevii* introgression lines against *T. timopheevii* cytoplasm

Summary

Wheat/*T. timopheevii* introgression lines were tested on CMS lines to localize genes in *T. timopheevii* that restore male fertility to hexaploid wheat with *T. timopheevii* cytoplasm. Characterization of the *T. timopheevii* chromosomes in these fertile lines was done by genotyping (KASP markers). Fertile lines carrying a single segment of *T. timopheevii* were detected. Chromosome 6G caused partial fertility restoration (1.31 seeds/spikelet) whereas chromosome $1A^t$ caused high fertility restoration (2.22 seeds/spikelet). Others fertile lines carried multiple segments of *T. timopheevii*. Others restorer factors were suspected on the chromosomes 2G, $4A^t$, 5G, $7A^t$ and 7G but further work is needed. The line with the highest fertility level carried seven different segments of *T. timopheevii* as well as the restorer allele of *Rf3* from 1BS of Chinese Spring.

VII.1. Introduction

VII.1.1. *T. timopheevii* genome

T. timopheevii is a tetraploid wheat (2n = 4x = 28, A^tA^tGG) originating from Georgia and domesticated from *T. araraticum* (Jakubz.) (Kilian et al., 2009; Mori et al., 2009). *T. araraticum* appeared less than 300,000 years ago and thus is considered as a younger species than the emmer species (Kilian et al., 2009; Mori et al., 2009) (refer to Chapter III.1.1.1). Many cytological studies were done to study the chromosome structure of *T. timopheevii* compared to wheat. The level of homoeologous chromosome divergence can be estimated by the frequencies of substitution or translocations between wheat and *T. timopheevii* chromosomes. Whereas wheat and *T. timopheevii* have common ancestors (i.e. the A/A^t genomes originated from *T. urartu* and B/G from *Ae. speltoides*) the low frequency of chromosome paring and the fertility of the wheat/*T. timopheevii* hybrids suggested a high degree of divergence between

their genomes (Brown-Guedira et al., 1996; Feldman, 1966; Leonova et al., 2011; Wagenaar, 1961). Chromosomes $1A^t$, $2A^t$, 2G, 3G, 5G and 6G show high frequencies of substitutions, whereas substitutions with chromosomes $3A^t$, $4A^t$, $6A^t$, $7A^t$ 1G, 4G and 7G are rare (Badaeva et al., 1991, 2000, 2010; Gordeeva et al., 2009). Wheat and *T. timopheevii* genomes diverged by several rearrangements which accumulated through evolution (Kilian et al., 2006). The G and B genomes are more diverged than the A^t and A genomes (Salina et al., 2006). Four species-specific translocations ($6A^tS$/1GS, 1GS/4GS, 4GS/$4A^tL$ and $4A^tL$/$3A^tL$) have been identified in the *T. timopheevii* genome as well as a paracentric inversion of the $6A^t$ chromosome (Jiang and Gill, 1994; Maestra and Naranjo, 1999; Rodriguez et al., 2000) (**Figure VII-1**). Species-specific translocations occurred at an early stage after the polyploid formation and could be the result of the genomic shock (Badaeva et al., 1995a; Feldman and Levy, 2012). These translocations were confirmed by the construction of *T. timopheevii* chromosome maps with SSR markers (Dobrovolskaya et al., 2009; Salina et al., 2006). However, some intraspecific translocations were found in particular *T. timopheevii* accessions such as the $5A^t$/3G, 1G/2G or 5G/6G translocations (Rodriguez et al., 2000). *T. timopheevii* shares with wheat the 4AL/5AL translocation which occurred in *T. urartu*, the diploid progenitor of the A and A^t genomes but not the 4AL/7BS translocation which took place in the tetraploid progenitor of wheat (Devos et al., 1995; Maestra and Naranjo, 1999).

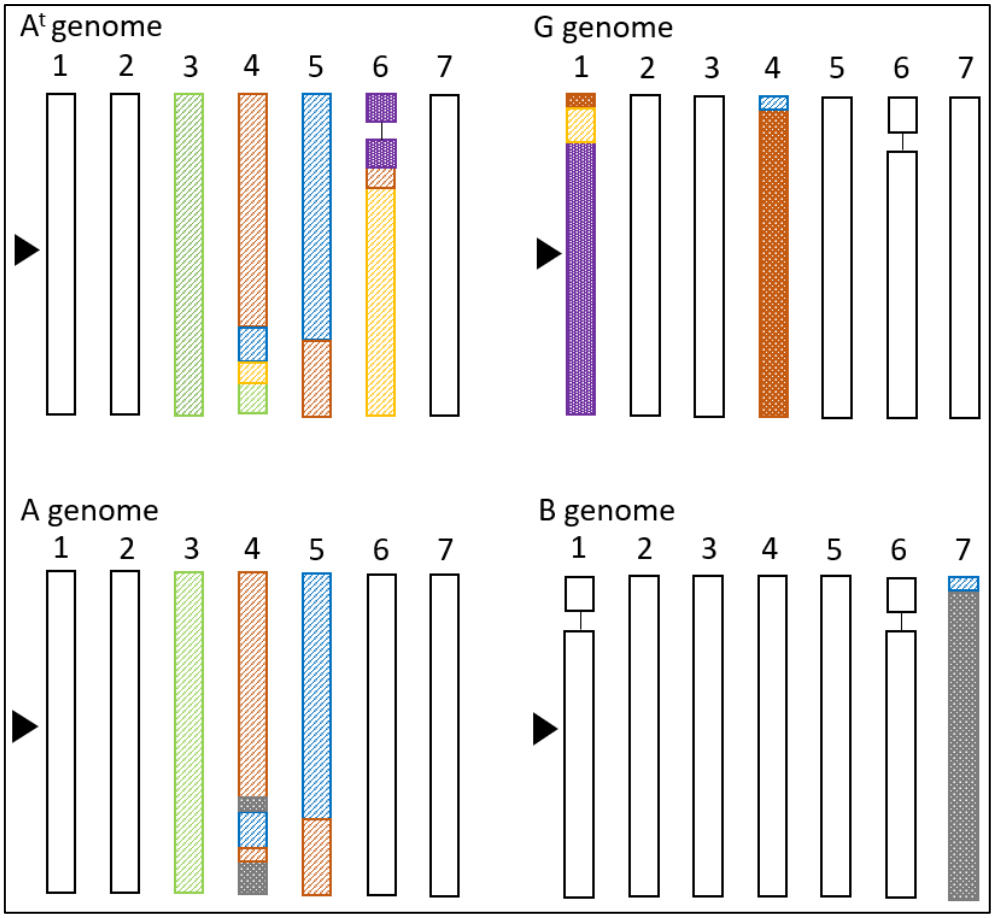


Figure VII-1 Schematic representation of chromosomal translocations in the *T. aestivum* A and B genomes and the *T. timopheevii* A^t and G genome
Centromere is shown by the black arrow. According to (Devos et al., 1995; Dobrovolskaya et al., 2009; Salina et al., 2006)

VII.1.2. *T. timopheevii* genome represents a huge reservoir of genes for wheat improvement

T. timopheevii has been of interest to breeders for a long time as its genomes contain a high number of valuable resistance genes (Friebe et al., 1996; Järve et al., 2002; Leonova et al., 2011). Resistance genes against stem rust (*Sr36*, *Sr37*, *Sr40*), powdery mildew (*Pm6*, *Pm27*, *Pm37*) and leaf rust (*Lr18* and *Lr50*) have been identified in the *T. timopheevii* genome (**Table VII-1**) (McIntosh et al., 2013). In addition Fusarium head blight resistance has been found in some accessions and transferred into spring wheat (Cao et al., 2009) with QTLs mapped on chromosome 5AL and 6A (Malihipour et al., 2016). Moreover, increased drought tolerance, cold resistance and baking qualities in wheat/*T.*

timopheevii introgression lines have also been reported (Mikó et al., 2015; Sokolov et al., 2017; Wan et al., 2002).

Table VII-1 Some resistance genes present on *T. timopheevii* genome

Locus	Chromosome	Reference
Sr36	2GS	(Allard and Shands, 1954; Nyquist, 1957)
Pm6	2GL	(Jørgensen and Jensen, 1973; McIntosh, 1991)
Sr40	2G	(Dyck and Friebe, 1993)
Lr50	2GL	(Brown-Guedira et al., 2003)
Sr37	4GS	(McIntosh and Gyarfas, 1971)
Lr18	5GL	(McIntosh, 1983)
Pm27	6G	(Badaeva et al., 1995b; Jørgensen and Jensen, 1973)
Pm37	$7A^t$	(Perugini et al., 2008)

Many studies have been carried out to transfer these traits to wheat through spontaneous recombination resulting in the production of numerous introgression lines. Common wheat was pollinated by *T. timopheevii* and the hybrid line was back-crossed and then selfed several times to stabilize the genome (Järve et al., 2000; Leonova et al., 2011). For example, the leaf rust resistance gene *Lr18* has been transferred from chromosome 5G to chromosome 5BL (McIntosh, 1983; Yamamori, 1994). The stem rust resistance gene *Sr36,* closely linked to the powdery mildew resistance gene *Pm6,* was transferred from chromosome 2G to chromosome 2B of wheat (Jørgensen and Jensen, 1973; Nyquist, 1957, 1962). These genes have been widely used in wheat cultivars (McIntosh et al., 1995; Purnhauser et al., 2011). More than 3,000 wheat varieties from all around the world were analysed and almost 600 of them were found to be carrying resistance genes *Sr36*, *Pm6* and/or *Lr18* of *T. timopheevii* (Martynov et al., 2018). However, it seems that one accession of *T. timopheevii*, D-357-1, was the main source of the resistance genes, reducing the genetic diversity brought into wheat. This low use of *T. timopheevii* potential in wheat pre-breeding could be the result of several

obstacles such as the sterility of the F_1 hybrids and the homoeology between the genomes preventing recombination events. The A^t and G genomes are partially homologous to the A and B genomes of wheat respectively. Transfer of genes from *T. timopheevii* to common wheat via homologous recombination is possible if the target gene is localized in a syntenic chromosome region. However, the two genomes diverged via the accumulation of translocations. Chromosomes of homoeologous groups 4A and 6A accumulated the highest number of differences as they are involved in species-specific translocations (Salina et al., 2006). For that reason, an introgression crossing programme has been started by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) using the *ph1* mutant.

VII.1.3. Methods to characterize *T. timopheevii* introgression

Development of high throughput characterization tools, such as molecular and cytogenetic markers, are essential to identify and efficiently track the introgressed segments. Cytogenetic studies have included C-banding, GISH and FISH (Badaeva and Gill, 1995; Badaeva et al., 1991, 1994, 2000, 2010, 2016; Jiang and Gill, 1994; Mikó et al., 2015; Rodriguez et al., 2000). Because of the similarity of the two genomes, GISH does not allow the distinction between the A and A^t genomes and between the B and G genomes. A variety of probes for FISH have been developed, e.g. Afa-family, pSc119.2, pTa71, $(GAA)_n$ (Adonina et al., 2015; Badaeva et al., 2015; Mikó et al., 2015). A karyotype of the two genomes of *T. timopheevii* was developed using the repetitive DNA probes pSc119.2 and Afa-family (Mikó et al., 2015). Each of the chromosomes of *T. timopheevii* can be distinguished and also differentiated to the wheat chromosomes as well (Sepsi et al., 2008). However, these methods do not allow a high throughput screening and are usually considered as confirmation/validation of the results from molecular marker screening. Large sets of molecular markers have been developed to identify segments of *T. timopheevii* transferred into the wheat genome. For example, SSR markers were used to map *Pm37* (Perugini et al., 2008), *Pm1* (Maxwell et al., 2009), *Lr18* (Leonova et al., 2010, 2011) and *Lr50* (Brown-Guedira et al., 2003). Some

wheat SSR markers were mapped on to the *T. timopheevii* chromosomes (Dobrovolskaya et al., 2009; Salina et al., 2006). These markers were used to develop a marker-assisted backcrossing (MAB) strategy to develop and characterise wheat/*T. timopheevii* introgression lines (Timonova et al., 2013). However, these markers do not allow a completey high throughput screening with high resolution of the lines produced. For that reason, more recently an Axiom® Wheat/Wild relative 35K SNPs array has been developed made up of SNPs showing polymorphism between wheat and ten of its wild relatives (including *T. timopheevii*) (Winfield et al., 2016). These SNP markers were converted to KASP markers by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham).

VII.1.4. *T. timopheevii* and hybrid wheat

Cytoplasm of *T. timopheevii* was reported to cause male sterility in wheat (Wilson and Ross, 1962) (refer to Chapter III.1.1.2). Its cytoplasm was considered promising as it caused stable and complete sterility with few deleterious effects on the phenotype (Tsunewaki et al., 1976, 1996; Wilson and Driscoll, 1983; Wilson and Ross, 1962). Moreover, some restorer genes have already been identified in *Ae. umbellulata*, rye and the wheat genomes (refer to Chapter III.1.1.3) (**Table VII-2**). For these reasons *T. timopheevii* cytoplasm has been the centre of interest of the CMS hybrid wheat programmes in the last century. However, no monogenic, total and stable restorer gene has been found upto now and transferred into the wheat genome which is the basic requirement for a commercial hybrid wheat programme.

Table VII-2 Restorer genes against *T. timopheevii* cytoplasm in different species genome

Species	Chromosome	Locus	Reference
Ae. umbellulata	6U	*Rf6*	(Kurauchi et al., 1977)
Rye	4RL	*Rfc4*	(Curtis and Lukaszewski, 1993)
Rye	6RL	*Rfc3*	(Curtis and Lukaszewski, 1993)
Wheat	1AS	*Rf1*	(Bahl and Maan, 1973)
Wheat	7D	*Rf2*	(Bahl and Maan, 1973)
Wheat	1BS	*Rf3*	(Bahl and Maan, 1973)
Wheat	6BS	*Rf4*	(Bahl and Maan, 1973)
Wheat	6D	*Rf5*	(Yen et al., 1969)
Wheat	5DS	*Rf6*	(Bahl and Maan, 1973)
Wheat	7B	*Rf7*	(Bahl and Maan, 1973)

T. timopheevii is considered as a natural carrier of *Rf* genes against its own cytoplasm. The G genome in particular is a potential source of *Rf* genes, as it comes from the S genome of *Ae. speltoides* which is the cytoplasm donor (Mori et al., 1997). Two dominant genes were detected in the *T. timopheevii* genome of the *T. timopheevii*/wheat cultivar Marquis hybrid back-crossed twice by Marquis. These two genes caused total restoration when they were both present in the CMS-line (Livers, 1964). However, wheat monosomic analysis suggested that one of the *Rf* genes was located on chromosome 1A (Robertson and Curtis, 1967), so it is not clear if the restorer genes came from *T. timopheevii* or the wheat genome.

By transferring the knowledge of *Rf* genes present in the wheat genome, localization of *Rf* genes in the *T. timopheevii* genome can be predicted (**Table VII-2**). For example, it has been shown that the *Rf1* gene present in the wheat genome came from *T. timopheevii* (Geyer et al., 2018). Moreover, chromosome 1G (*Rf3* located on 1B of wheat), chromosome 6G (*Rf4* on 6B) and 7G (*Rf7* on 7B) are potential restorer chromosomes. Localization of these loci in the *T. timopheevii* genome also depends on the genome rearrangements

which occurred during wheat's evolution. Moreover, as the D genome of wheat is the result of the hybridization of the A and B genome (Marcussen et al., 2014; Sandve et al., 2015), some *Rf* genes located in the D genome of wheat could be present on the *T. timopheevii* genome. However, we also did not eliminate the possibility of the existence of new restorer loci/alleles, which were not discovered in wheat genome.

VII.1.5. Aim and objectives of the chapter

The main hypothesis of the chapter was that the first source of *Rf* genes against *T. timopheevii* cytoplasm is *T. timopheevii* itself. These genes have been not exploited until now because of the absence of wheat/*T. timopheevii* translocation lines with the genes of interest and the lack of characterization tools. The aim of the chapter was therefore:

- To test wheat/*T. timopheevii* introgression lines produced at the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) for their ability to restore *T. timopheevii* cytoplasm.
- To characterize the fertile F_1 plants using the KASP markers developed in-house.

VII.2. Material and Methods

VII.2.1. Plant material

Three different hexaploid wheat varieties carrying *T. timopheevii* cytoplasm were used as the female parent. Two lines were spring type and one line was winter type. The CMS lines were provided by Limagrain and were at the BC_6 generation. Wheat/*T. timopheevii* introgression lines were used as pollinators. A large panel of wheat/*T. timopheevii* introgression lines were developed by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham). The initial cross consisted in the pollination of Chinese Spring *ph1* mutant or Paragon *ph1* mutant by one accession of *T. timopheevii* (PI P95-99.1-1). A back cross programme was carried out by using common wheat as the recurrent male parent (Paragon, Pavon 76 or Highbury). The Axiom® Wheat/Wild relative 35K SNP array was used to genotype the introgression lines and based on this

genotyping, 142 different introgression lines (at generations ranging from BC_2F_2 to BC_4F_1), carrying at least one of the chromosomes 1G, 6G or 7G were selected. The complete pedigree of the lines used is shown in Appendix 3.

VII.2.2. Crossing programme

Figure VII-2 shows the crossing scheme chosen. To produce the F_1 generation, CMS wheat was pollinated by wheat/*T. timopheevii* introgression lines. As CMS lines was used as the female lines there was no need for emasculation. Each head of the CMS line was covered with a bag before the opening of the spikelets to avoid pollen contamination (refer to Chapter II.1.4). Once the stigmas were fluffy and receptive, heads were pollinated with wheat/*T. timopheevii* introgression line pollen. After pollination, heads were bagged to protect them.

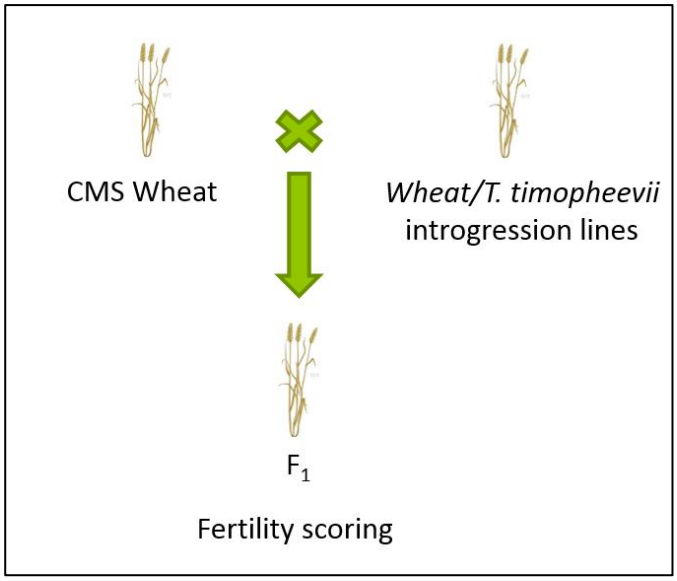


Figure VII-2 Crossing scheme to screen the ability of wheat/*T. timopheevii* introgression lines to restore *T. timopheevii* cytoplasm

VII.2.3. Seed germination

Germination of the seeds was carried out according to the protocol (refer to Chapter II.1.1). Once the seed germinated, it was sown into soil (John Innes compost No. 2). Plants were put into vernalisation for four weeks (at 8°C with 12 hours light per day) and then transferred in to the glasshouse (at 21°C, 16 hours light per day).

VII.2.4. Assessment of the fertility restoration and position of the seeds along the spike

To score the male fertility restoration, one to three heads of the F_1 were bagged prior to anthesis and harvested after ripening. For each selfed spike, the number of seeds as well as the number of spikelets were counted and both were averaged by plant. The seed set of a plant was defined as the number of seeds divided by the number of spikelets. A plant without any selfed seed was considered as male sterile, whereas a plant with at least one seed was considered as male fertile. Common wheat was used as a control.

As each spike was composed by a different number of spikelets, it was divided into six portions (from the base to the apex). The position of each seed within the spike was recorded. The average number of seeds/spikelet for each portion was calculated for each spike. A global average of all the spikes for each portion was calculated.

VII.2.5. KASP markers genotyping

Out of the markers produced and validated, those polymorphic between the different wheats and the wild *T. timopheevii* accession were selected leading to a set of 233 markers. 231 of the markers were assigned to a *T. timopheevii* linkage group according to the previous genotyping data done by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham). From these 231 markers, a total of 141 markers were selected to cover the fourteen linkage groups of the *T. timopheevii* genome. Genomic DNA extraction of the fertile F_1 was done by the method explained in Chapter II.2. PCRs were run by the protocol explained in Chapter II.4.2. Each mixture was composed of 5 µL of DNA, 2.43 µL of dH_2O, 2.50 µL of ROX PACE mix and 0.068 µL of primers mix. The details of the PCR cycle were given in Chapter II.4.2.

VII.2.6. *Rf3* locus genotyping

The restoration ability of the wheats involved in the crossing scheme was tested by pollinating spring and winter type CMS lines. Whereas all the hybrids

between the CMS line and Paragon, Pavon 76 or Highbury were male sterile, some hybrids with Chinese Spring showed fertility restoration when the CMS line was winter type (refer to Chapter III.3.5). Chinese Spring is known to carry the restorer gene *Rf3* on chromosome 1BS (Kučera, 1982). For this reason, fertile F_1 lines were genotyped with SNP markers, developed in-house by Limagrain, to screen for the presence of the restorer allele of the *Rf3* locus located on chromosome 1BS of wheat (cultivar Chinese Spring).

VII.3. Results

VII.3.1. F_1 generation production

A total of 254 CMS heads were pollinated producing 1875 F_1 seeds. However, crosses done with nineteen introgression lines did not set any seeds, mainly because of the poor pollen production of these lines. F_1 seed was selected to cover all the combinations of CMS x wheat/*T. timopheevii* introgression lines. A total of 621 F_1 were sown, of which 510 germinated and reached the adult stage.

VII.3.2. Fertility restoration scoring

A total of 49 F_1 plants set selfed seed and thus were considered as fertile, whereas the other 461 F_1 plants were sterile. The fertility level ranged from 0.01 to 2.74 seeds/spikelet while the controls set an average of 3.20 seeds/spikelet (**Figure VII-3**). 35% of the lines had a very low level of restoration with less than 0.5 seeds/spikelet, 31% of the F_1 lines set between 0.5 and 2 seeds/spikelet, 24% of the fertile lines had a medium fertility level with a seed set ranging from 1 to 2 seeds/spikelet and 10% of the lines set more than 2 seeds/spikelet. The shape of some fertile heads of different fertility levels are presented in **Figure VII-4**. Moreover, the variation of fertility within a plant could be important (Figure presented in Appendix 4). For example, the fertility level of line 28 ranged from 0.17 to 1.47 seeds/spikelet and the fertility level of line 30 ranged from 0.29 to 1.65 seeds/spikelet.

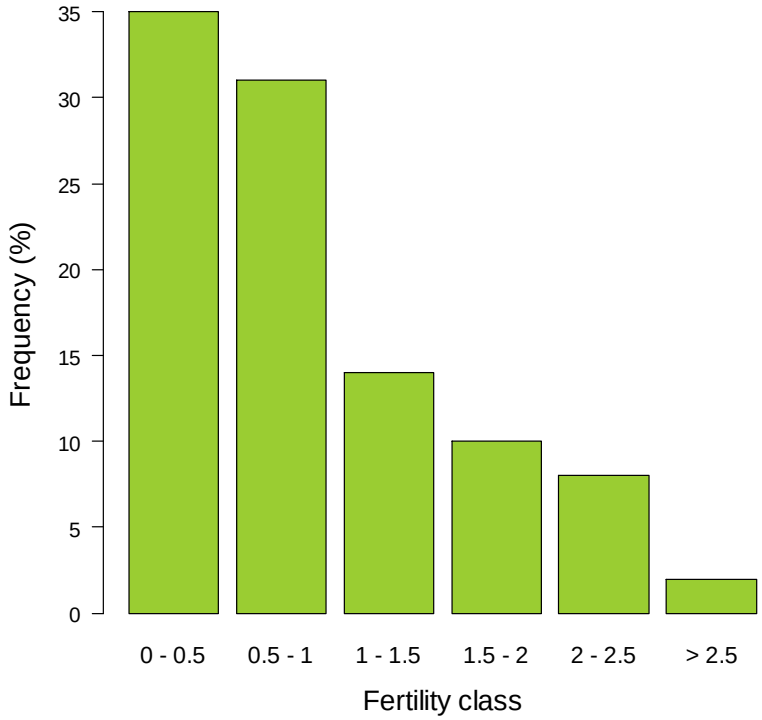


Figure VII-3 Distribution of fertility of the 49 F_1 lines



Figure VII-4 Head shape of fertile lines

Line 23 set 0.60 seeds/spikelet, line 26 0.78 seeds/spikelet, line 48 2.36 seeds/spikelet and line 49 2.74 seeds/spikelet

The distribution of the seeds along the spike is represented in **Figure VII-5**. As the length of the spike varied between individuals, the spike was divided into six equal portions. The number of seeds/spikelet ranged from 0 to 4 seeds. The highest number of seeds was observed in the second and third sections of the spike with a median value of 1.66 and 1.52 seeds/spikelet respectively. The lowest number of seeds was observed at the apex of the spike. More than half of the heads did not set any seed in spikelets of section 5, while three quarters of spikelets did not set seeds in section 6. Those heads setting seed right up to the apex of the spike were the ones with the highest seed set.

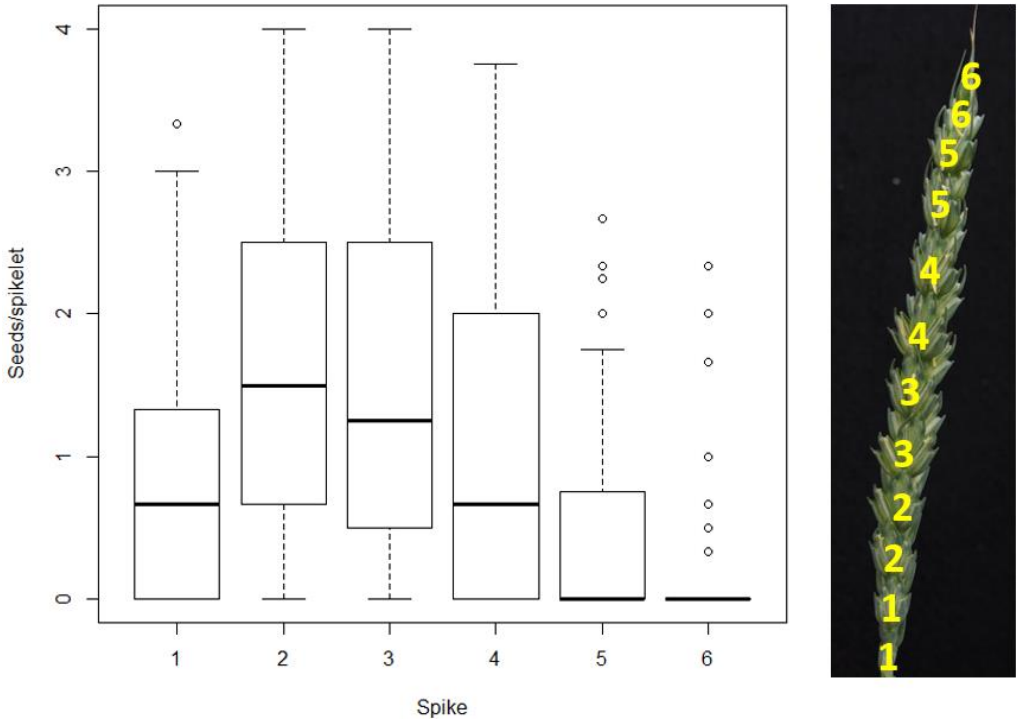


Figure VII-5 Distribution of the seed set along the spike

The x axis number indicates the spike section: 1 is the first section from the base of the spike and 6 is the apex as shown on the photo on the right

In the box plots, the boundary of the box closest to zero indicates the 25th percentile, a black line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles. Points above the whiskers indicate outliers outside the 90th percentiles.

VII.3.3. Characterization of the fertile lines with KASP markers

Out of the 231 KASP markers available, a total of 141 markers were run on the 49 fertile F_1 lines (**Table VII-3**). One hundred and eleven markers amplified

correctly with 65 markers located on the A^t genome and 48 markers located on the G genome.

Table VII-3 Number of KASP markers available, tested and confirmed for each linkage group of *T. timopheevii*

Linkage group	No. of polymorphic markers	No. of marker tested	No. of markers which amplified
$1A^t$	11	9	7
$2A^t$	17	12	10
$3A^t$	8	7	4
$4A^t$	12	7	6
$5A^t$	14	9	6
$6A^t$	12	10	9
$7A^t$	10	9	8
1G	14	10	8
2G	25	11	9
3G	28	7	6
4G	21	8	6
5G	15	13	9
6G	23	15	11
7G	21	14	12
Total	231	141	111

Genotyping results are presented in **Figure VII-6**. The KASP results were consistent between siblings (delimited by the bold framework in **Figure VII-6**) and also with the genotyping of the previous generation carried out with the Axiom® Wheat/Wild relative 35K SNPs array leading to the conclusion that the data obtained with the KASP markers were accurate. However, two markers were repositioned, one from $7A^t$ to $6A^t$ and one from 5G to $4A^t$. No fertile lines with linkage group 1G were detected. Eight lines carried a segment of 6G chromosome (16%) and fourteen lines carried a segment of 7G (35%) (**Table VII-4**). A wide range of segments of other linkage groups were detected in the

fertile lines involving chromosomes $1A^t$, $2A^t$, 2G, 3GS, $4A^t$, 4G, 5G, $6A^t$ and $7A^t$. In particular, chromosomes $1A^t$, 2G and $7A^t$ were present in 73%, 73% and 59% of the lines respectively. Four lines carried a single segment of *T. timopheevii*. Lines 35 and 47 carried a complete $1A^t$ chromosome and lines 36 and 38 most of chromosome 6G. Other lines carried many different fragments, up to a maximum of 7 (lines 49, 7, 3, 11 and 5). The highest number of introgressions was detected when the male parent was at the less advanced stage (i.e. BC_2F_2 compared to BC_4F_1). The majority of the *T. timopheevii* fragments detected were large, covering most of the linkage group.

Table VII-4 Proportion of each *T. timopheevii* linkage group in the fertile lines

Linkage group	**Percentage of lines carriers**
$1A^t$	73% (36/49)
$2A^t$	20% (10/49)
$4A^t$	14% (7/49)
$6A^t$	14% (7/49)
$7A^t$	59% (29/49)
2G	73% (36/49)
3G	4% (2/49)
4G	6% (3/49)
5G	10% (5/49)
6G	16% (8/49)
7G	39% (19/49)

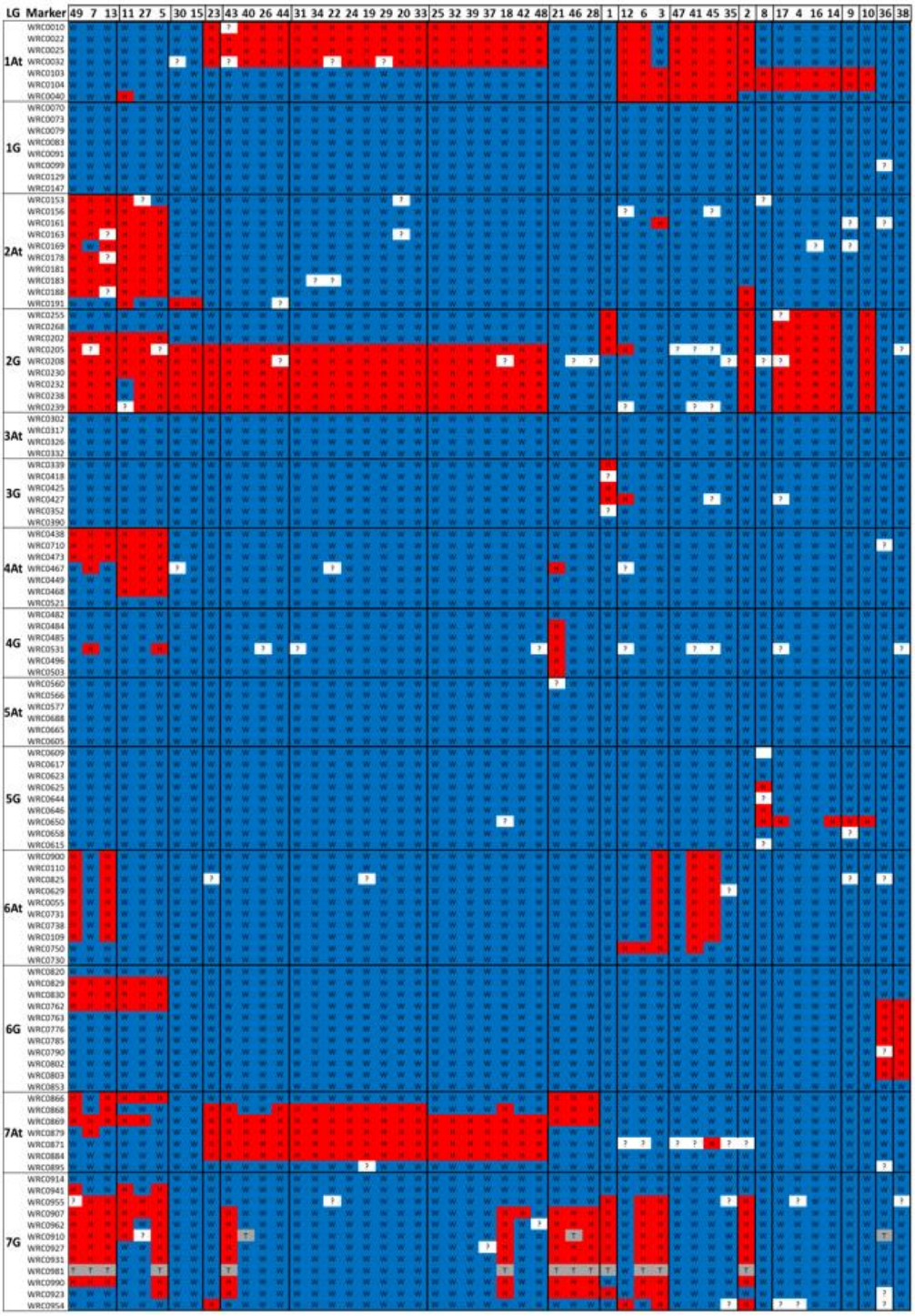


Figure VII-6. Graphical representation of the KASP genotyping of the fertile lines

Markers are ordered by linkage groups and are scored as “W” for wheat allele (blue colour), “H” for heterozygous (red colour) and “T” for *T. timopheevii* allele (grey allele). Missing values are indicating by “?” (white colour). Lines are regrouped by male parent lines (bold framework), from the less advanced to the more advanced generation

VII.3.4. Combination of fertility scoring and genotyping results

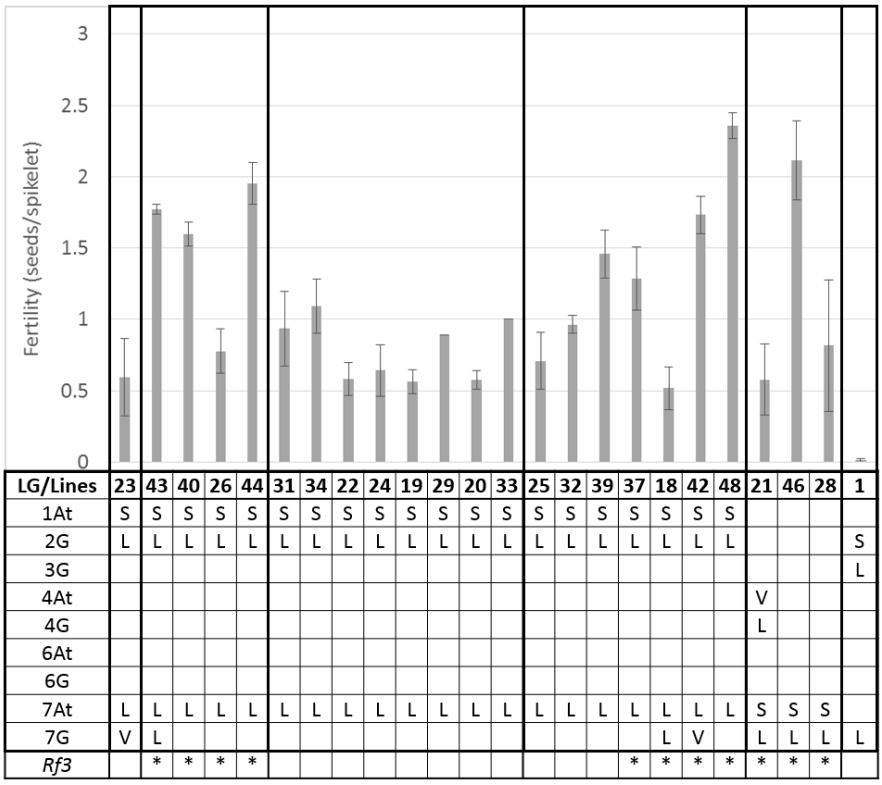
Figure VII-7 shows the genotyping results combined with the fertility restoration score for each fertile line. The lines were regrouped by their male parent (wheat/*T. timopheevii* introgression lines). In addition to the KASP genotyping, results of the presence of restorer alleles of the *Rf3* locus (on chromosome 1BS of wheat) were added.

VII.3.4.1. *Presence of a single segment of T. timopheevii*

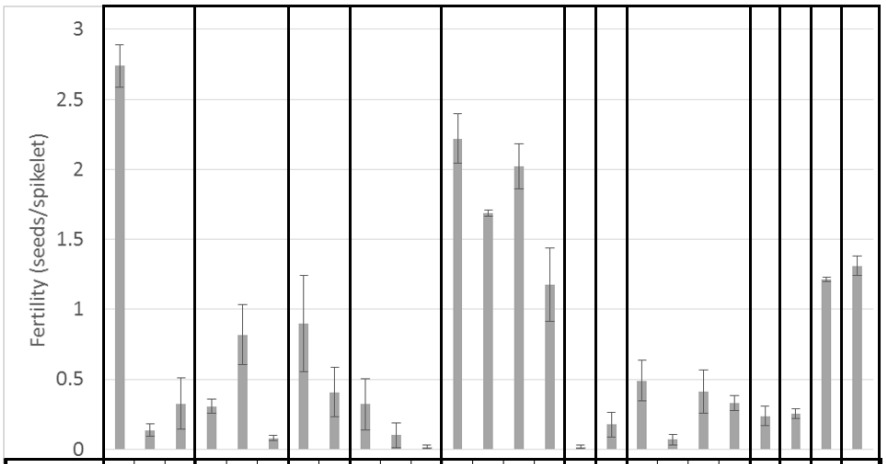
A single introgression is the ideal situation to isolate and characterize a restorer factor. Only four lines carrying a unique segment of *T. timopheevii* were detected. Lines 35 and 47 with the complete chromosome $1A^t$ set 1.18 and 2.22 seeds/spikelet respectively. Lines 36 and 38 carried a large segment of chromosome 6G and had a seed set of 1.21 and 1.31 seeds/spikelet respectively. These four lines, 35, 36, 38 and 47, did not carry the restorer alleles of *Rf3* from Chinese Spring.

VII.3.4.2. *Presence of multiple segments of T. timopheevii*

The remaining 41 fertile lines carried more than one segment of *T. timopheevii*. A total of nineteen combinations were detected with various lengths of segment introgressed. In addition, fifteen of the fertile lines carried the restorer allele of *Rf3*. The fertility observed in these fifteen lines could thus either be the result of the *Rf3* gene or a restorer gene present on *T. timopheevii*.



LG/Lines	23	43	40	26	44	31	34	22	24	19	29	20	33	25	32	39	37	18	42	48	21	46	28	1
1At	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S				
2G	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L				S
3G																								L
4At																					V			
4G																					L			
6At																								
6G																								
7At	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	S	S	S	
7G	V	L																L	V		L	L	L	L
Rf3		*	*	*	*												*	*	*	*	*	*	*	



LG/Lines	49	7	13	11	27	5	30	15	12	6	3	47	41	45	35	2	8	17	4	16	14	9	10	36	38
1At				V					F	F	S	F	F	F	F	L	S	S	S	S	S	S	S		
2At	L	L	L	F	L	L	V	V			V					S									
2G	L	L	L	S	L	L	L	L	V							F		F	F	F	F		F		
3G									V																
4At	S	S	S	L	L	L																			
4G		V				V																			
5G																	S	V			V	V	V		
6At	L		L						V	V	F		L	L											
6G	S	S	S	S	S	S																		L	L
7At	S	S	S	V	V	V								V											
7G	L	L	L	S	S	L			V	L	L					L									
Rf3	*			*	*	*																			

Figure VII-7 Combination of fertility and genotyping results

Genotypes with the same male parent are regrouped in a bold framework. Presence of the *T. timopheevii* genome was coded with the following letters: “F” for a complete chromosome, “S” for a segment of a chromosome, “L” for a large segment and “V” for a very small segment. Presence of restorer allele of *Rf3* is indicated by a *.

VII.3.4.3. *Conclusion*

Two different segments of *T. timopheevii* were successfuly isolated, a complete chromosome $1A^t$ and a large segment of chromosome 6G. The maximum fertility scored in the lines carrying only one of these chromosomes was 2.22 and 1.31 seeds/spikelet respectively. Other fertile lines carried segments from two to seven different chromosomes of *T. timopheevii*. Some of these lines also carried the restorer allele of the *Rf3* gene. The most fertile lines carried various segments of *T. timopheevii* genome. For example, line 49 had a fertility of 2.74 seeds/spikelet, line 48 had a fertility of 2.36 seeds/spikelet and line 46 had a fertility of 2.11 seeds/spikelet. In some cases, variation was observed within the F_1 siblings carrying the same fragments of *T. timopheevii*. For example, line 47 carried almost double the amount of seeds/spikelet compared to line 35. Lines 28 and 46 carried the same segments of *T. timopheevii* genome and set 0.82 and 2.11 seeds/spikelet respectively. However, both lines were carriers of the restorer allele of the *Rf3* locus. In contrast, lines 25 and 48 carried the same segments of the *T. timopheevii* genome and set 0.71 and 2.36 seeds/spikelet respectively, but while the restorer allele of *Rf3* was present in line 48, it was absent in line 25.

VII.4. Discussion

T. timopheevii cytoplasm is considered as the most promising for commercial hybrid wheat programmes. Several *Rf* genes have been found in related species such as rye, *Ae. umbellulata* and wheat varieties (Bahl and Maan, 1973; Curtis and Lukaszewski, 1993; Kurauchi et al., 1977). However, a complete monogenic and stable *Rf* gene in different environments and genotypes has not been found yet. *T. timopheevii* is considered as a natural carrier of *Rf* genes against its own cytoplasm. The G genome, in particular, is a potential source as it comes from the S genome of *Ae. speltoides* which is the cytoplasm donor of *T. timopheevii* (Mori et al., 1997). The existence of two dominant *Rf* genes have already been reported (Livers, 1964). Many wheat/*T. timopheevii* introgression lines have been developed in order to transfer various interesting traits into the wheat genome (McIntosh et al., 1995). However, the potential of *T.*

timopheevii has not been exploited until now due to two reasons. The first is the partial homology between wheat and the *T. timopheevii* genomes, i.e. A with A^t and B with G. If the trait of interest is located in a region of the *T. timopheevii* genome which is not homologous to the wheat genome, the probability of a recombinant event and thus transfer of the gene from *T. timopheevii* genome to wheat is very low because of the presence of the *Ph1* locus on chromosome 5B of wheat. The second reason for the under exploitation of *T. timopheevii*'s potential is the lack of high throughput tools to characterize the introgression lines produced. FISH techniques have been successfully developed but they are highly time consuming. Several SSR markers have also been developed, but these markers do not allow a completey high throughput screening with high resolution of the lines produced (Brown-Guedira et al., 2003; Dobrovolskaya et al., 2009; Leonova et al., 2011; Salina et al., 2006). To overcome these two barriers the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) started a crossing programme with accession PI P95-99.1-1 of *T. timopheevii*. Female parents consisted of two different wheat varieties, Chinese Spring and Paragon, with the *ph1* locus deleted, in order to increase the probability of recombination events between *T. timopheevii* and wheat. Chinese spring *ph1b* was the first mutant developed (Sears, 1977). Paragon was used as the recurrent parent as it was considered as an elite variety (Angus et al., 2011). This material has been characterized by the Axiom® Wheat/Wild relative 35K SNPs array (Winfield et al., 2016).

F_1 production

A panel of 142 wheat/*T. timopheevii* introgression lines was selected as potential donors of *Rf* genes (lines were selected on the basis of the genotyping carried out on their own parents) . Three specific linkage groups were targeted by transposing the knowledge of the *Rf* genes present in the wheat genome against *T. timopheevii* cytoplasm: chromosomes 1G, 6G and 7G. These chromosomes are potential carriers of the three dominant genes *Rf3*, *Rf4* and *Rf7* respectively. However, other *T. timopheevii* linkage groups were present

in the selected lines. The offspring of these lines were used as pollen donor on CMS-lines with *T. timopheevii* cytoplasm. A large number of wheat/*T. timopheevii* introgression lines were tested as it was assumed that some of the lines would have lost the segment of *T. timopheevii* (due to segregation in the gametes). A total of 1875 F_1 seeds were produced. 510 F_1 plants were scored for their fertility. 49 F_1 set self-seeds ranging from 0.01 to 2.74 seeds/spikelet, wheras selfed common wheat set an average of 3.20 seeds/spikelet. 66% of the lines had a very low fertility level with less than one seed/spikelet, 24% a medium fertility (between 1 and 2 seeds/spikelet) and 10% set more than 2 seeds/spikelet. No complete restoration was observed in comparison with the control. This partial fertility, however, might not only be the result of the restoration system but it could also be explained by genomic instability caused by the presence of the *T. timopheevii* genome translocated into wheat preventing correct pairing during meiosis because of the lack of complete homology (Gordeeva et al., 2009; Leonova et al., 2011). Genotyping results did not give information about the composition of the wheat genome. In our study, male parents used were generations ranging from BC_2F_2 to BC_4F_1, while in a previous study, genome instability was still detected in lines at generation BC_1F_{20} (Gordeeva et al., 2009). This could cause an incomplete genome in the F_1 plants, thus impacting the phenotype. Importantly, fertility variation between the different spikes of an individual was observed, highlighting the weak stability of the restoration. Moreover, a decreasing gradient of fertility from the base to the apex of the spike was observed, a result supported by previous observations (Virmani and Edwards, 1983). Seeds were observed in the more terminal spikelets only in the most fertile spikes.

Genotyping results

In order to characterize the introgression lines, the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) developed KASP markers polymorphic between wheat and *T. timopheevii*. Out of the 141 markers run, 111 amplified correctly. These markers were spread through all the fourteen linkage groups of *T. timopheevii*. All the 49 fertile lines carried at least one

segment of *T. timopheevii* which came from various linkage groups: $1A^t$, $2A^t$, 2G, 3GS, $4A^t$, 4G, 5G, $6A^t$, 6G, $7A^t$ and 7G. Unfortunately, while the segment is heterozygous, the genotyping does not enable the identification of whether the introgression is an addition, a substitution or a translocation nor into which linkage group of wheat the segment has been introgressed. The majority of the lines carried a segment from at least one of the linkage groups $1A^t$, 2G or $7A^t$ (linkage groups present in 59% to 73% of the lines). While a single introgression is the ideal situation to isolate and characterize a restorer factor, only four lines carried a unique segment of *T. timopheevii*. Two lines had the complete $1A^t$ chromosome and the other two a large segment of chromosome 6G. The other lines carried between two and seven different *T. timopheevii* segments. Out of the targeted linkage groups, only 6G and 7G were detected in the fertile lines. The absence of linkage group 1G could be explained by the loss of the 1G linkage group in the lines being tested, as the Axiom® Wheat/Wild relative 35K SNPs array genotyping was carried out on the parents of the wheat/*T. timopheevii* lines used as male parents. Two segregation events thus occurred, the first one by selfing the genotyped parental line and the second one by pollinating the CMS lines, increasing the probability of the lost *T. timopheevii* genome. The other hypothesis is the absence of a restorer factor on chromosome 1G, i.e. the *Rf3* locus could have evolved specifically in the wheat genome. Two other linkage groups were targeted, 6G and 7G, which were present in eight and fourteen fertile lines respectively. Thirty-five fertile lines did not carry one of these two targeted linkage groups. This means that other restorer genes were present in the fertile lines produced. In addition, a restorer factor could be carried from the wheat genome. Whereas all the hybrids between the CMS line and Paragon, Pavon 76 or Highbury were male sterile, some hybrids with Chinese Spring showed fertility restoration when the CMS line was winter type (refer to Chapter III.3.5). Indeed Chinese Spring carries the restorer gene *Rf3* on chromosome 1BS (Kučera, 1982). For that reason, fertile lines were screened for the presence of the restorer allele of *Rf3* and fifteen of them found to be carriers of this allele.

It should be noted that these are only preliminary results. An increase in the number of lines studied is required to have more reliable data. In addition, it is possible that some of the regions of *T. timopheevii* were not covered by the genotyping according to the low density of the markers, i.e. more segments might have been present than were detected.

Chromosome $1A^t$

Two lines carried a single chromosome of *T. timopheevii* which was identified as chromosome $1A^t$. These lines did not carry the restorer allele of *Rf3*. Thus, chromosome $1A^t$ of *T. timopheevii* is able to restore its cytoplasm. Chromosome $1A^t$ resulted in a fertility ranging from 1.18 to 2.22 seeds/spikelet. A parallel could be drawn with the restorer gene located on wheat chromosome 1AS, named as *Rf1* (Bahl and Maan, 1973). It has been shown that the *Rf1* gene present in the wheat genome came from *T. timopheevii* (Geyer et al., 2018). Lines with this single *Rf1* gene present set less than 1.5 seeds/spikelet (Geyer et al., 2018). The allele present in the lines produced from accession PI P95-99.1-1 of *T. timopheevii* seems to have a higher expressivity than the one already introgressed into the wheat cultivars. However, the expression of fertility is not only the result of the presence of one restorer gene. Modifier loci play an essential role in the fertility restoration as they significantly influence the restorer gene expression, which can be prevented in their absence (Robertson and Curtis, 1967). Several wheat chromosomes have been identified as carriers of modifier loci, depending on the pedigree (Du et al., 1991; Geyer et al., 2018; Maan et al., 1984; Robertson and Curtis, 1967; Tahir and Tsunewaki, 1969). It is thus possible that the F_1 producing 2.22 seeds/spikelet carried a better combination of modifier loci than the lines studied previously. However, some lines with chromosome $1A^t$ produced less than 0.5 seeds/spikelet (for example lines 23 and 6). This low fertility could be explained by either the genomic instability of the F_1 or by the presence/absence of modifier genes which do not allow the expression of the restorer gene even if it is present (Geyer et al., 2018; Robertson and Curtis, 1967; Würschum et al., 2017b). It has been shown that *ph1* mutant wheat,

which was used as the initial parent for the wheat/ *T. timopheevii* introgression lines, has an unstable karyotype with aneuploidy and wheat intergenomic translocations (Sanchez-Moran et al., 2001). All the line carrying the complete $1A^t$ chromosome do not express a high fertility level. For example, line 6 set only 0.10 seeds/spikelet. These results emphasize the complexity of the restoration system. Furthermore, Robertson and Curtis (1967) and Maan et al. (1984) demonstrated that environmental factors could also influence the detection of restorer and modifier loci.

The presence of a strong restoration gene on chromosome $1A^t$ could explain its over-representation in the fertile lines (present in 73% of the lines). The precise localization of the restorer gene on chromosome $1A^t$ is unknown as no line carrying a single small segment of this chromosome was detected. However, it has been reported that chromosome $1A^t$ does not differ structurally from chromosome 1A (Maestra and Naranjo, 1999; Rodriguez et al., 2000). In this study, the line carrying a segment covering the long arm of the $1A^t$ chromosomes restored fertility to a lower level than the line with a segment covering the short arm of the chromosome. However, segments of other chromosomes of *T. timopheevii* were present in the first line. Although we can assume that the restorer gene is located on the short arm of chromosome $1A^t$, this hypothesis needs further work to be confirmed.

Chromosome 6G

Two lines had a single segment of *T. timopheevii* which was identified as a large part of chromosome 6G. These lines did not carry the restorer allele of *Rf3* and thus chromosome 6G is a carrier of a restorer gene which causes a restoration ranging from 1.21 to 1.31 seeds/spikelet. This gene could be the same locus as *Rf4* on chromosome 6BS of wheat (Bahl and Maan, 1973). However, it is difficult to confirm that these two loci are identical without comparing the marker haplotype for the genomic regions of these two *Rf* genes. *Rf4* restores fertility against *T. timopheevii* cytoplasm to a lower level compared to *Rf1* (Geyer et al., 2018).

According to KASP results, the segment involved covered most of 6G. No other lines carried the same segment of chromosome 6G. Further studies on a larger population are needed to confirm these preliminary results. Six other lines did carry a segment of chromosome 6G but this segment only overlapped the short arm of the segment present in lines 36 and 38 (with the large segment). Various other segments of *T. timopheevii* were also present in these six lines (fertility ranging from 0.08 to 2.74 seeds/spikelet) as well as the restorer allele of *Rf3* in four of them. It could be interesting to use the overlapping strategy developed by Sears, (1972), i.e. by crossing the two lines carrying the overlapping segments, to isolate this overlapped segment and conclude if it is a carrier of the restorer gene.

Chromosome 7G

The presence of a restorer gene on chromosome 7G was also predicted as chromosome 7B of wheat carries the *Rf7* gene (Bahl and Maan, 1973). However, a chromosome 7G single introgression line was not produced. Moreover, most of the lines with chromosome 7G also carried the restorer locus of *Rf3* from Chinese Spring. Chromosome 7G was present in lines 49 and 46, which showed high fertility (more than 2 seeds/spikelet). However, six other segments of *T. timopheevii* and the restorer allele of *Rf3* were present in line 49 while line 46 was composed of the short arm of chromosome $7A^t$ and the restorer allele of *Rf3*. Regarding the different combinations it seems too early to draw a conclusion about the ability of the 7G chromosome to restore the fertility of *T. timopheevii* cytoplasm.

Chromosome $7A^t$

Two thirds of the fertile lines carried a segment of chromosome $7A^t$. No restorer gene has previously been reported on chromosome 7A of wheat. An absence of lines carrying only this chromosome prevent any conclusion about its ability as a restorer of fertility.

Chromosome 2G

Chromosome 2G was found in 73% of the fertile lines. The high occurrence of chromosome 2G could be the result of its preferential transmission through the generations. A high frequency of the presence of chromosome 2G in substitution lines has previously been reported. The hypothesis proposed was that chromosome 2G of *T. timopheevii* carries a gametocidal gene (Brown-Guedira et al., 1996). Gametocidal genes have been identified on chromosome 2 of several Aegilops species: *Ae. speltoides*, *Ae. sharonensis*, *Ae. cylindrica*, *Ae. longissima* (Endo, 1990, 2007) (refer to Chapter I.7). However, all these gametocidal genes do not have the same strength and the same mode of action and in milder cases, gametes lacking the *Gc* chromosome suffer slight chromosome damage, can be fertilized and the *Gc* chromosomes are not always selectively retained. The tetraploid condition of *T. timopheevii* could explain the incomplete selection of its gametocidal gene. The presence of non-centromeric translocations observed in some of the lines could be a consequence of the junction of broken chromosomes (Badaeva et al., 1995a). Gametocidal genes can be of interest if it is linked to an good agronomic trait which will be retained in the successive generations resulting in easier introgression for the breeder (Endo, 1990). For example, chromosome 2G harbours genes for multiple disease resistance (*Sr36*, *Pm6, Sr40* and *Lr50*) (McIntosh et al., 2013). However, presence of the gametocidal gene can cause female and male sterility problems (Tsujimoto and Tsunewaki, 1985b). The presence of chromosome 2G could thus reduce the fertility, leading to the conclusion that the restoration of fertility is only partial in comparison with the control. If the presence of a gametocidal gene on chromosome 2G is confirmed by additional studies, this chromosome will have to be removed from the fertile lines to reach a non-reduced fertility. The elimination of chromosome 2G is not impossible as some lines without it have been identified.

Alternatively, the high occurrence of chromosome 2G in the fertile lines could be interpreted as the presence of a restorer or a modifier gene. Two results support this hypothesis. The first is the presence of a large segment of

chromosome 2G plus the presence of a very small segment of chromosome $2A^t$ in lines 15 and 30 setting 0.41 and 0.90 seed/spikelet respectively. These two lines did not carry the restorer allele of *Rf3*. This leads to the conclusion that a restorer locus is present either on chromosome $2A^t$ or on chromosome 2G. The presence of a restorer locus on chromosome 2G is confirmed by lines 4 and 16. These two lines carry a segment of the long arm of chromosome $1A^t$ (the restorer gene is supposed to be located on the short arm of this chromosome) and the complete 2G chromosome. The fertility ranged from 0.07 to 0.41 seeds/spikelet. No restorer gene has previously been reported on chromosome 2B.

Chromosome 5G

Line 8 carries a segment covering the long arm of chromosome $1A^t$ and a segment of chromosome 5G. The restorer locus on chromosome $1A^t$ should not be present according to the hypothesis expressed in the paragraph above about chromosome $1A^t$. In addition, no restorer allele of the *Rf3* gene was detected. This means that chromosome 5G might be able to restore fertility. Line 8 had a seed set of 0.18 seeds/spikelet. No restorer locus has been detected on chromosome 5B of wheat. However, chromosome 5DS carries a restorer locus, named as *Rf6* (Bahl and Maan, 1973). It has been shown that the D genome is the result of the hybridization between the A and B genomes (Marcussen et al., 2014; Sandve et al., 2015) (to Chapter I.2.2).

Chromosome $4A^t$

A segment covering the short arm of chromosome $4A^t$ was detected in six fertile lines. However, the presence of other segments of *T. timopheevii* were detected in these lines, as well as the restorer allele of *Rf3* in four them. Thus no conclusions can be drawn regarding the fertility restoration ability of chromosome $4A^t$ without further work.

Monogenic or multigenic restoration?

Total restoration under monogenic control is the ideal situation for an efficient breeding program (Sage, 1972). Five F_1 line produced more than 2 seeds/spikelet considered as high fertility. However, out of the lines showing high fertility only line 47 carried a single segment of *T. timopheevii*. This line set 2.22 seeds/spikelet which represents 70% of the fertility scored for the control. In reality, as discussed above, the restoration is not only the result of restorer genes but modifier genes are also essential to completey restore fertility. These genes do not directly cause fertility, but they inhibit or enhance the effectiveness and the expression of the restorer gene. Modifier loci have been identified on different wheat chromosomes depending on the genotype (Chen, 2003; Du et al., 1991; Geyer et al., 2018; Maan et al., 1984; Robertson and Curtis, 1967; Tahir and Tsunewaki, 1969; Würschum et al., 2017b). Thus it can be assumed that modifier loci are also present in *T. timopheevii*. This increases the difficulty of the production of the perfect male restorer. Parental combinations are essential as the modifier loci can be carried by the male parent as well as by the CMS line.

It has been suggested that a complete and stable fertility necessarily involves several *Rf* genes in wheat (Johnson and Patterson, 1973). The four other most fertile lines carried multiple segments of *T. timopheevii* and three of them also had the restorer allele of *Rf3* from 1BS of Chinese Spring. Line 49, which set 2.74 seeds/spikelet, had segments of chromosomes $2A^t$, 2G, $4A^t$, $6A^t$, 6G, $7A^t$ and 7G. In an analogy with the *Rf* genes known in the wheat genome, line 49 is a potential carrier of *Rf4* (6G) and *Rf7* (7G). In addition, our results showed that chromosomes 2G and $7A^t$ might carry restorer loci. Line 48 carried a segment of chromosome $1A^t$ (*Rf1*), 2G and $7A^t$, in addition to the restorer allele of *Rf3*. Line 46 had the restorer allele of *Rf3* plus segments of chromosome $7A^t$ and 7G (*Rf7*?). Line 45 carried the complete $1A^t$ chromosome (*Rf1*), a segment of chromosome $6A^t$ and a small segment of $7A^t$.

Conclusion and perspectives

T. timopheevii is considered as the most promising for commercial hybrid wheat programmes as its cytoplasm has very few deleterious effects on the wheat phenotype. However, a monogenic, total and stable restorer (in both different environments and genotypes) has not been found yet. Wheat/*T. timopheevii* introgression lines produced by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) were tested on a CMS-line with the cytoplasm of *T. timopheevii*. A total of 49 F_1 showed fertility restoration. The fertility level ranged from 0.01 to 2.74 seeds/spikelet. Two thirds of the lines set less than 1 seed/spikelet. The fertile lines were characterized with the 111 KASP markers developed in house by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham). By the use of single introgression lines it was possible to show that chromosomes $1A^t$ and 6G of *T. timopheevii* were able to partially restore fertility. The analogy with the *Rf* genes present in the wheat genome led to the hypothesis that *Rf1* and *Rf4* loci are present in *T. timopheevii*. Other chromosomes of *T. timopheevii* were also suspected to carry restorer factors, i.e. chromosomes 2G, 5G, $7A^t$ and 7G. Moreover, not all the genome of *T. timopheevii* was tested. Some of the lines carried the restorer allele of the major restorer gene *Rf3*, located on 1BS of wheat. The fertility variation observed within lines carrying the same segments of *T. timopheevii* revealed the low stability of the introgression, the probable genomic instability of the lines and the influence of the genetic background (modifier loci).

However, the results presented in this chapter are preliminary results, with further investigations needed to confirm them. The first step will be to increase the size of the population studied to produce more reliable data. The study of a higher number of single introgression lines will allow a complete characterization of the restoration capacity of chromosomes $1A^t$ and 6G. In addition, a back-cross programme could be carried out to stabilize the genome in order to increase the fertility. However, attention has to be paid to the modifier loci which are essential to reach complete fertility restoration. The same backcross strategy could be applied to the fertile lines carrying several

segments of *T. timopheevii*. The segments should segregate through the generations leading to their individualization. New restorer loci might be discovered in this way. Our preliminary results suggest the presence of *Rf* loci on chromosomes 2G, 5G, $7A^t$ and 7G. The reduction of the size of the segments of *T. timopheevii* would also be interesting, to reduce linkage drag and thus possible negative effects on the fertility scored. An overlapping fragment strategy has been successfully developed in the past (Sears, 1972). For example, two lines with two different and overlapping fragments of chromosome $1A^t$ with the *Rf* gene in the middle, could be crossed. Recombination could occur between the two chromosomes in the overlap regions and thus offspring of the cross could carry reduced segments of chromosome $1A^t$ with the *Rf* gene and without the deleterious genes.

According to the results of this chapter and previous studies, a combination of different *Rf* genes seems to be the only realistic option to reach a total and stable restoration. A combination of *Rf* genes can be achieved by crossing two lines carrying two different *Rf* locus. However, this strategy implies a more complex and longer-term breeding scheme. The availability of KASP markers will help to make this process quicker due to easier screening for the segments.

VIII. Chapter 8: Study of the restoration of *T. timopheevii* cytoplasm by *Ae. sharonensis*

Summary

Wheat/*Ae. sharonensis* addition lines were produced to be to localize genes in *Ae. sharonensis* that restore male fertility to wheat with *T. timopheevii* cytoplasm. *Ae. sharonensis* carries a gametocidal chromosome which is preferentially transmitted to the next generation. In order to remove this gametocidal chromosome, a knocked-out mutant T4BS.4BL-4S^{sh}L translocation line was used in the crossing scheme. Fertile addition lines were characterized by cytogenetics methods (GISH) combined to genotyping (KASP markers). Two new restore chromosomes were identified. Chromosome 5S^{sh} restored the fertility to a level of 1.75 seeds/spikelet and chromosome 6S^{sh} to a level of 2.02 seeds/spikelet.

VIII.1. Introduction

VIII.1.1. *Aegilops sharonensis* genome

Ae. sharonensis (or Sharon grass) originated from Israel and southern Lebanon. It is considered as a highly diverse species (Olivera and Steffenson, 2009; Olivera et al., 2010). Although *Ae. sharonensis* is generally considered autogamous, outcrossing has been commonly observed (Dvořák et al., 1998b). It is an annual diploid species (2n = 2x = 14) belonging to the Sitopsis section of the genus *Aegilops* together with *Ae. speltoides*, *Ae. longissima Ae. searsii* and *Ae. bicornis* (van Slageren, 1994). Its genome has been named as S^{sh} or S^{l} (Teoh and Hutchinson, 1983). Cytological studies by C-banding demonstrated that the genome of *Ae. sharonensis* maintained the chromosome structure of the B genome of hexaploid wheat without any evidence of any chromosomal rearrangements (Friebe and Gill, 1996; Maestra and Naranjo, 1997). Nevertheless, they cannot be considered as homologous genomes. *Ae. sharonensis* has been proposed as a putative donor of the B genome based on meiotic paring and morphological analysis (Kushnir and Halloran, 1981, 1982).

This hypothesis has been rejected because of the low paring frequency between the wheat B-genome chromosomes and those of *Ae. sharonensis* (Fernández-Calvin and Orellana, 1993; Maestra and Naranjo, 1997; Riley et al., 1958) and *Ae. speltoides* or a closely related extinct species is now considered as the strongest candidate for the B genome donor (Huang and et al., 2002; Salse et al., 2008). It has also been suggested that the *Ae. sharonensis* genome is more closely related to the D genome than the B genome (Maestra and Naranjo, 1997; Marcussen et al., 2014; Zhang et al., 2001).

VIII.1.2. *Ae. sharonensis* and wheat improvement

VIII.1.2.1. *Traits of interest detected in Ae. sharonensis*

Ae. sharonensis is a large reservoir of genes for wheat improvement for disease resistance and abiotic stress tolerance. High levels of resistance to powdery mildew, leaf rust, stem rust, stripe rust, spot blotch and tan spot have been reported (Anikster et al., 2005; Gerechter-Amitai and Loegering, 1977; Gill et al.; Olivera et al., 2007). A single dominant gene conferring resistance to leaf rust has been mapped on chromosome $6S^{sh}$ (Olivera et al., 2013). More particularly, *Ae. sharonensis* has received attention as a source of resistance to the Ug99 race of wheat stem rust which is one of the main threats to wheat production (Olivera and Steffenson, 2009; Olivera et al., 2007; Scott et al., 2014). Two resistance genes against the Ug99 race have been mapped on chromosomes $1S^{sh}$ and $5S^{sh}$ of accession AEG-1644 (Yu et al., 2017). Important diversity for resistance to leaf rust, stem rust and powdery mildew was found within *Ae. sharonensis* accessions (Olivera et al., 2008, Amitai and Loegering 1997). Moreover, *Ae. sharonensis* has been identified as salt, aluminium and boron tolerant (Manyowa and Miller, 1991; Xu Xing (Agricultural Coll. et al., 1993) and also shows a high transpiration efficiency (Waines et al., 1993).

VIII.1.2.2. *Barriers to the exploitation of the potential of Ae. sharonensis*

Despite the great potential of the genome of *Ae. sharonensis* to improve common wheat, the existence of two barriers has prevented the production of wheat/*Ae. sharonensis* introgression lines.

VIII.1.2.2.1. The gametocidal chromosome

The presence of a gametocidal (*Gc*) chromosome in *Ae. sharonensis,* which is retained through the generations, prevents the production of a complete set of wheat/*Ae. sharonensis* addition or substitution lines (Maan, 1975; Miller et al., 1982). Two *Gc* chromosomes have been detected on chromosome $2S^{sh}$ and $4S^{sh}$ named as *Gc1-S^{l}1* and *Gc2* respectively (Endo, 1982, 1985; Finch et al., 1984b; McIntosh et al., 1998; Miller et al., 1982) (refer to Chapter I.7). *Gc* loci are present in several *Sitopsis* species (Endo, 1990). A dual function model was proposed to explain the mechanism responsible for the preferential transmission of the *Gc* chromosome associated with two phenotypes (Endo, 1990). The first phenotype is denominated as "breaker" and induces chromosome breakages in the gametes. The second is the "inhibitor" phenotype which prevents chromosome breakage in the gametes carrying the *Gc* chromosome. The *Gc2* gene of chromosome $4S^{sh}$ has an intense gametocidal action, gametes without the *Gc* chromosome will suffer severe chromosome breakage and become sterile (Endo, 1982, 1985, 1990; Maan, 1975; Nasuda et al., 1998; Tsujimoto and Tsunewaki, 1984a). Only the gametes with the *Gc2* will be functional. This gene is transmitted to the next generation at a frequency of 98% by both male and female gametes with no influence of the wheat genetic background (King et al., 1991). In order to circumvent this barrier, an ethyl methylsulfonate (EMS) induced mutant of the breaker element of the *Gc2* gene has been produced (Friebe et al., 2003). The gene producing the breaker element has been knocked-out, whereas the gene producing the inhibitor element remains intact. This mutation is dominant, the heterozygous mutant (*Gc2*/*Gc2*^{mut}) exhibits a normal transmission, with the production of functional gametes and completey fertile spikes. Half the

gametes contain a functional *Gc2* copy and the other half the mutated *Gc2* copy, which both produce the inhibitor element. The mutation has been transferred into a Chinese Spring genetic background as a homoeologous distal translocation $4S^{sh}L$ of *Ae. sharonensis* in the wheat 4BL chromosome (T4BS.4BL-$4S^{sh}$L). The breaker element has been mapped to the distal end of the long arm of chromosome $4S^{sh}$ (Endo, 2007; Knight et al., 2015). Eighteen candidates have been identified as the potential breaker element (Grewal et al., 2017).

VIII.1.2.2.2. The *Ph1* locus

The partial homology of the *Ae. sharonensis* genome with the wheat genome prevents the exchange of chromatin between the two genomes due to the existence of the *Ph1* locus on chromosome 5BL of wheat (Okamoto, 1957; Riley and Chapman, 1958; Sears and Okamoto, 1958) (refer to Chapter I.6.2). The use of a *ph1* mutant is required to induce homoeologous pairing and enhance the probability to produce wheat/*Ae. sharonensis* recombinants.

VIII.1.2.3. *Successful production of wheat/Ae. sharonensis introgression lines*

By using the T4BS.4BL-$4S^{sh}$L translocation line with the mutated *Gc* locus and the *ph1* mutant some interesting wheat/*Ae. sharonensis* introgressions have been produced. A leaf rust resistance gene *LrS12* (*Lr56*) and a stem rust resistance gene *YrS12* (*Yr38*) were detected in accession 174 from Israel and transferred into wheat by backcrossing the haploid hybrid wheat/*Ae. sharonensis* (Marais et al., 2003) six times. The recurrent wheat parent, line W84-17, was considered as a neutralizer of the gametocidal gene. The translocated chromosome, with the two resistance genes, has been identified as chromosome $6S^{sh}$ of *Ae. sharonensis* with a terminal segment of its long arm replaced by the corresponding segment of wheat 6AL (Marais et al., 2006). The 6AL-$6S^{sh}$L.$6S^{sh}$S translocation line was crossed with Chinese Spring *ph1b* to reduce the size of the fragment of *Ae. sharonensis* (Marais et al., 2010). 53 recombinant lines carrying *Lr56* were produced. Cytogenetic and genotyping

studies showed that three of these recombinants carried a small distal fragment of chromosome $6S^{sh}$ (Somo et al., 2016). Other $6B.6S^{sh}$ recombinants resistant to leaf and stem rust were produced by using the $Gc2^{mut}$ line and *ph1b* mutant in the first cross generation using accession TH548 (Millet et al., 2014). It is not clear if these genes are identical to *Lr56* and *Yr38* already identified by Marais et al. (2010). The same chromosome engineering technologies were used to transfer resistance genes to African stem rust from the *Ae. sharonensis* accessions AEG-1644 and AEG-2172 into common wheat resulting in the production of 1S.1B, 1S.1D and 5S.5B translocation lines (Millet et al., 2017).

VIII.1.3. Characterization of wheat/*Ae. sharonensis* introgression lines

Efficient characterization tools are essential to optimize the utilisation of *Ae. sharonensis* in a breeding programme. *Ae. sharonensis* can be detected in a wheat background by using cytogenetic methods. GISH analysis successfully detected the presence of translocated chromosomes (Grewal et al., 2017; Knight et al., 2015; Somo et al., 2016). Different specific patterns for some of the chromosomes were observed with the probe pSc119.2, e.g. a strong signal on chromosome $6S^{sh}$, but no complete karyotype has been established (Badaeva et al., 1996; Kwiatek et al., 2013). However, cytogenetic methods cannot be considered as high throughput characterization tools as they are highly time consuming with low information resolution. Various molecular markers have been successfully developed to identify the location and the size of the introgressed fragment. For example, SSR and DArT markers were used to characterize the resistant recombinants (Marais et al., 2006, 2010; Millet et al., 2014; Somo et al., 2016). More recently, the ultra-high density Axiom® Wheat 80K SNP array was used to identify translocation lines resistant to stem rust (Millet et al., 2017).

VIII.1.4. *Ae. sharonensis* and hybrid wheat

The Sitopsis section of the Aegilops genus is composed by five species including *Ae. speltoides* and *Ae. sharonensis*. Like *T. timopheevii* and wheat, *Ae.*

speltoides carries in its mitochondrial DNA a sequence equivalant to *orf256*, a chimeric gene which expresses a 7 kDa protein, confirming *Ae. speltoides* as the cytoplasm donor to both *T. timopheevii* and wheat (Hedgcoth et al., 2002; Song and Hedgcoth, 1994b). The genome of *Ae. speltoides,* and more broadly the genomes of the Sitopsis species, are thus potential carriers of restorer genes against the cytoplasm of *T. timopheevii*. Like *Ae. speltoides*, *Ae. sharonensis* carries a strong gametocidal gene, *Gc2*, on chromosome $4S^{Sh}$ (Endo, 1982, 1985; Finch et al., 1984b; Miller et al., 1982). However, the breaker element of *Gc2* was knocked out and a wheat/*Ae. sharonensis* T4BS.4BL-$4S^{sh}$L translocation line with the *Gc2* element mutated was produced (Friebe et al., 2003) allowing the production of wheat/*Ae sharonensis* introgression lines without chromosome $4S^{sh}$ (Marais et al., 2010, Milet et al., 2014). For that reason, we decided to study the genome of *Ae. sharonensis* as a potential carrier of restorer genes against *T. timopheevii* cytoplasm.

VIII.1.5. Aim and objectives of the chapter

The aim of this chapter was to test the ability of the genome of *Ae. sharonensis* to restore the cytoplasm of *T. timopheevii*. For that purpose, the T4BS.4BL-$4S^{sh}$L translocation line with the *Gc* breaker element mutated was used to remove the *Gc* chromosome. The fertile progenies were characterized by cytogenetic and molecular markers.

VIII.2. Material and methods

VIII.2.1. Plant material

An *Ae. sharonensis* amphidiploid S55/2008 in a Chinese Spring background obtained from the Germplasm Resource Unit**,** JIC, was used as the donor of the *Ae. sharonensis* genome. Three different wheat cultivars have been used: the T4BS.4BL-$4S^{sh}$L translocation line (CS/*Gc* mutant) with the mutated allele of the *Gc* gene transferred into a Chinese Spring background (Friebe et al., 2003), a spring CMS line with the cytoplasm of *T. timopheevii* provided by Limagrain and their maintainer lines (the same variety as the CMS line with *T. aestivum* cytoplasm).

VIII.2.2. Crossing programme

Figure VIII-1 explains the crossing programme followed to test the genome of *Ae. sharonensis* on the cytoplasm of *T. timopheevii*. The CMS line and the wheat/*Ae. sharonensis* amphidiploid were pollinated by the CS/*Gc* mutant line. The F_1 resulting from the first crosses (①) were pollinated by the F_1 of the second crosses (②). The progeny of these crosses (③) were screened for fertility. The fertile lines were crossed with the maintainer line. The F_2 (④) and BC_1 (⑤) plants were also screened for their fertility.

In parallel, CMS lines were pollinated by the CS/*Gc* mutant line and non-mutant CS as controls to confirm the absence of restorer genes in the wheat background.

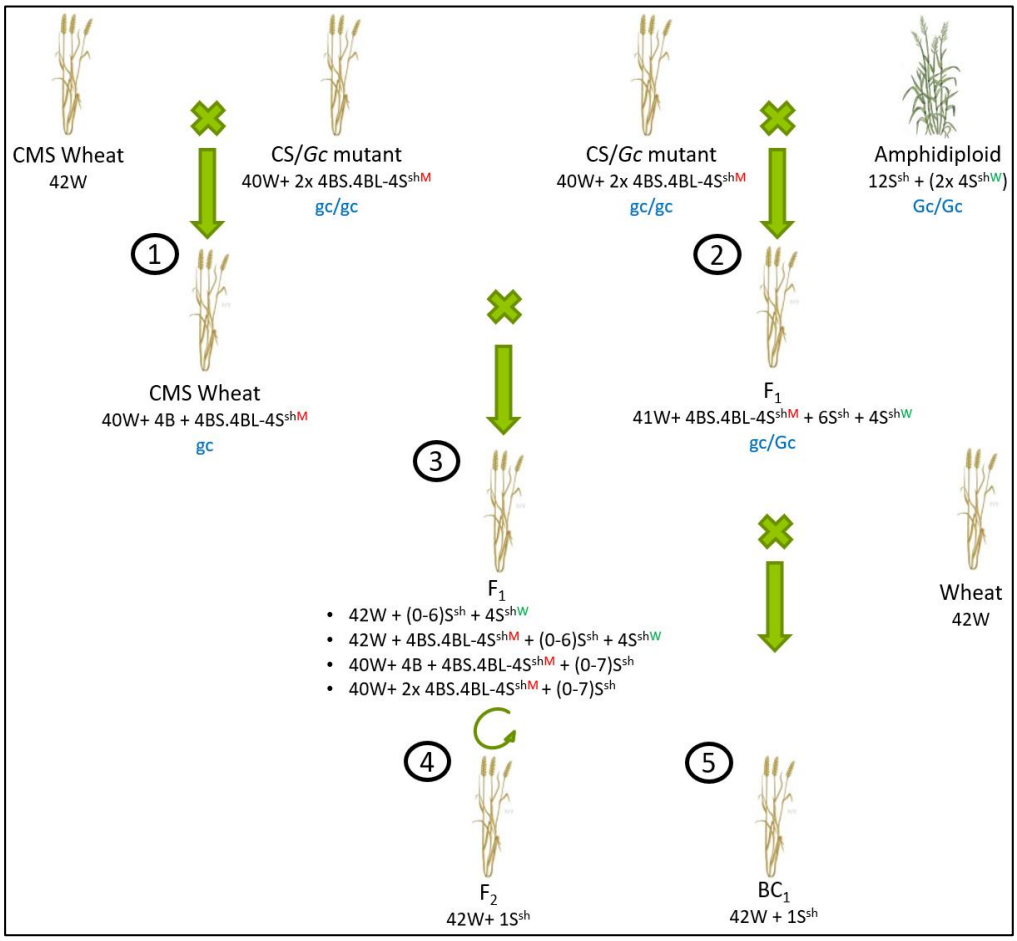


Figure VIII-1. Crossing scheme followed to obtain wheat/*Ae. sharonensis* introgression lines restoring the cytoplasm of *T. timopheevii*

S^{shW} is the wild allele (*Gc*) of the *Gc* gene on chromosome $4S^{sh}$ and $4S^{shM}$ is the mutated allele (*gc*) of the *Gc* gene (Friebe et al., 2003).

VIII.2.3. Seed germination

Germination of the seeds was carried out according to the established protocol (refer to Chapter II.1.1). As the F_1 seeds (Amphidiploid x CS/*Gc* mutant (②)) were shrivelled, embryo culture was used to enhance the germination success. The seeds were sterilised with 1 mL 5% ClNaO (10% solution-sigma Aldrich, Appendix 1), the embryo was rescued and placed on a culture medium (MS + 2% sucrose + 1% agarose, Appendix 1) under sterile conditions. Once the seed germinated, it was sown into soil (John Innes compost No. 2). The wheat/*Ae. sharonensis* amphidiploid were put into vernalisation for six weeks, the following generations and the wheats were put for four weeks (at 8°C with 12 hours light per day) and then transferred to the glasshouse (at 21°C, 16 hours light per day).

VIII.2.4. Fertility restoration scoring

To score the male fertility restoration, one to three heads per plant were bagged prior to anthesis and harvested after ripening. For each selfed spike, the number of seeds as well as the number of spikelets were counted and both were averaged by plant. The seed set of a plant was defined as the number of seeds divided by the number of spikelets. A plant without any selfed seed was considered as male sterile, whereas a plant with at least one seed was considered as male fertile. Common wheat was used as a control.

VIII.2.5. Cytogenetic analysis

Preparation of metaphase spreads was described in Chapter II.3.3. Slides were probed as follows. Single colour GISH was carried out using total genomic DNA of common wheat as blocking DNA and total genomic DNA of *Ae. sharonensis* labelled by nick translation with Alexa Fluor 488-5-dUTP (Invitrogen; C11397) (green) as probe. The slides were counterstained with DAPI.

VIII.2.6. KASP markers

A total of 1000 KASP markers polymorphic between the wheat cultivars and the ten wild relatives (one of which is *Ae. speltoides*) have been developed by

the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) in collaboration with LGC. These 1000 KASP markers have been validated on 5000 genotypes, including the wild accession of *Ae. sharonensis* used here and the wheat parents. Out of the markers validated, those polymorphic between the different wheats and the wild *Ae. sharonensis* accession were selected leading to a set of 158 markers. Using the high synteny within the species of the Sitopsis section of the genus *Aegilops*, 54 of the markers were assigned to an *Ae. sharonensis* linkage group according to the previous genotyping data done by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) on the wheat/*Ae. speltoides* introgression lines. From these 54 markers, a total of 36 markers were selected to cover the seven linkage groups of the *Ae. sharonensis* genome. Genotyping was carried out on the newly developed fertile lines and the 6AL-6S^{sh}L.6S^{sh}S translocation line produced by Marais et al., (2006). Genomic DNA extraction was done by the method explained in Chapter II.2. PCRs were run by the protocol explained in Chapter II.4.2. Each mixture was composed of 5 µL of DNA, 2.43 µL of dH_2O, 2.50 µL of ROX PACE mix and 0.068 µL of primers mix (refer to Chapter I.4.2).

VIII.3. Results

VIII.3.1. Germplasm production

Table VIII-1 presents the results of the crosses carried out upto generations F_2 and BC_1. The pollination of the CMS lines by the CS/*Gc* mutant resulted in a good seed set (6.40 seeds/head) producing 64 non-shrivelled seeds. A total of 30 seeds were germinated successfuly (①). However, the pollination of the wheat/*Ae. sharonensis* amphidiploid by the CS/*Gc* mutant resulted in the production of 74 shrivelled seeds. An average of 1.35 seeds were set per head crossed. A total of 45 seeds were sown but only 28.89% germinated (②). Eighteen heads of ① were pollinated by ② producing 17 seeds, mostly non-shrivelled. The limiting factor during these crosses was the low pollen production of ②. Twelve F_1 seeds were sown but only five of them germinated (③). Three of the F_1 were male fertile and produced 129 F_2. Only these three

F1 were pollinated by the maintainer line. An average of 10.33 seeds were set per head crossed producing a total of 93 BC_1 seeds. 68.85% and 60.87% of the F_2 and BC_1 seeds sown germinated. However, five F_2 plants died during development, giving a total of thirty-seven adult plants. A total of 330 F_3 seeds were produced but no BC_1 plants set seeds. Fertile F_2 plants were pollinated by the maintainer line producing 21 seeds.

Table VIII-1 Germination and success of the crosses for each generation

For the meaning of column headings refer to **Figure VIII-1**

	CMS x CS/*Gc* Mutant	**Amphidiploid x CS/*Gc* mutant**	①	②	③ **(F_1)**	④ **(F_2)**	⑤ **(BC_1)**
Seeds sown	-	-	30	45	12	61	23
Germination rate	-	-	100%	28.89%	41.67%	68.85%	60.87%
Crosses made	10	55	18		9	4	0
Crossed seeds	64	74	17		93	21	0
Shrivelled seeds	0%	100%	17.65%		0%	0%	-
Seeds/head	6.40	1.35	0.94		10.33	5.25	-
Cross fertility	100%	45.46%	38.89%		88.89%	50%	-
Selfed-seeds	-	-	-		129	330	0

VIII.3.2. Fertility scoring

VIII.3.2.1. *Fertility of the F_1 generation*

Five F_1 plants were scored for their male fertility. Three of them set selfed seeds with similar levels of fertility restoration (**Table VIII-2**). The average fertility ranged from 0.44 to 0.53 seeds/spikelet (the control set 2.2 seeds/spikelet). Variation in the expression of the fertility within a plant was observed. For example, the less fertile head of F_1.1 set 0.44 seeds/spikelet while the more fertile head set 0.95 seeds/spikelet.

Table VIII-2 Fertility level (seeds/spikelet) of the fertile F_1

Line	Fertility	Minimum	Maximum
F_1.1	0.44	0.06	0.95
F_1.2	0.49	0.05	0.83
F_1.3	0.53	0.13	0.88

VIII.3.2.2. *Fertility of the BC_1 generation*

Out of the fourteen BC_1 plants scored for their fertility, none set selfed-seeds. All the BC_1 plants were thus considered as male sterile.

VIII.3.2.3. *Fertility of the F_2 generation*

A total of thirty-seven F_2 plants were screened for their fertility. Thirteen of them set at least one selfed-seed and thus were considered as fertile. The fertility level ranged from 0.02 to 2.02 seeds/spikelet (**Figure VIII-2**). Lines 1 to 10 expressed a low fertility restoration level with less than 0.5 seeds/spikelet whereas lines 11 to 13 were partially to highly fertile with more than 1.5 seeds/spikelet. The wheat used as control set 2.6 seeds/spikelet.

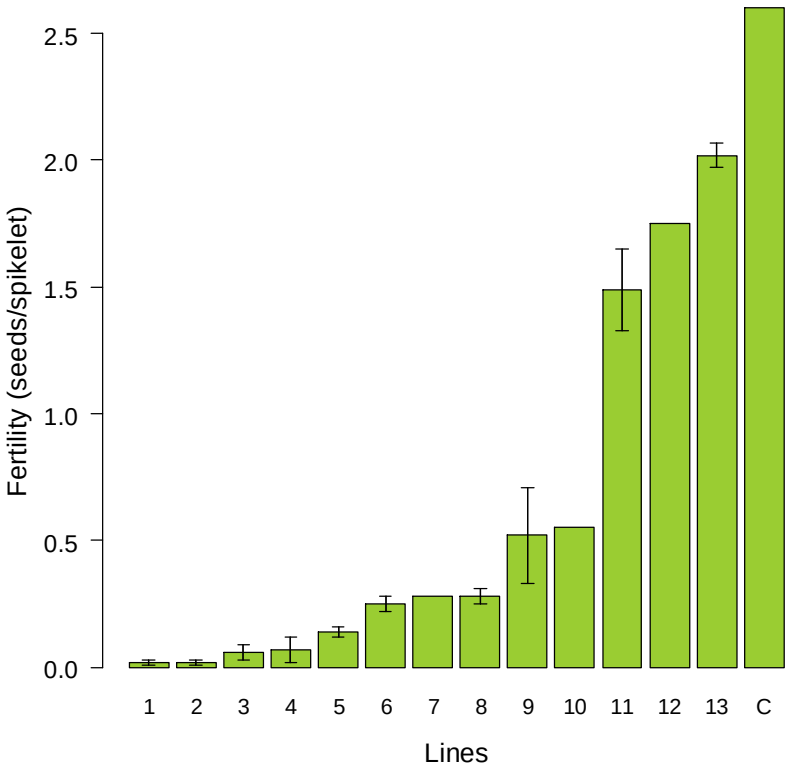


Figure VIII-2 Distribution of the self-fertility level of the F_2 lines

F_2 genotypes were numbered from 1 to 13, C designated the control (wheat)

VIII.3.2.4. *Fertility of control*

The F_1 plants resulting from the pollination of the spring CMS lines by both the CS/*Gc* mutant and the normal CS did not set any selfed-seeds and thus were all male sterile.

VIII.3.3. Characterisation by GISH

VIII.3.3.1. *Genomic composition of the F_1 generation*

Single-colour GISH was carried out successfully on the five F_1 lines. One to three chromosomes of *Ae. sharonensis* were added to the complete genome of wheat (i.e. 42 chromosomes) (**Table VIII-3** and **Figure VIII-3**).

Table VIII-3 Genomic composition of the F_1 lines

Line	No. of *Ae. sharonensis* chromosome	No. of wheat chromosome	Total
F_1.1	2	42	44
F_1.2	2	42	44
F_1.3	1	42	43
F_1.4	1	42	43
F_1.5	3	42	45

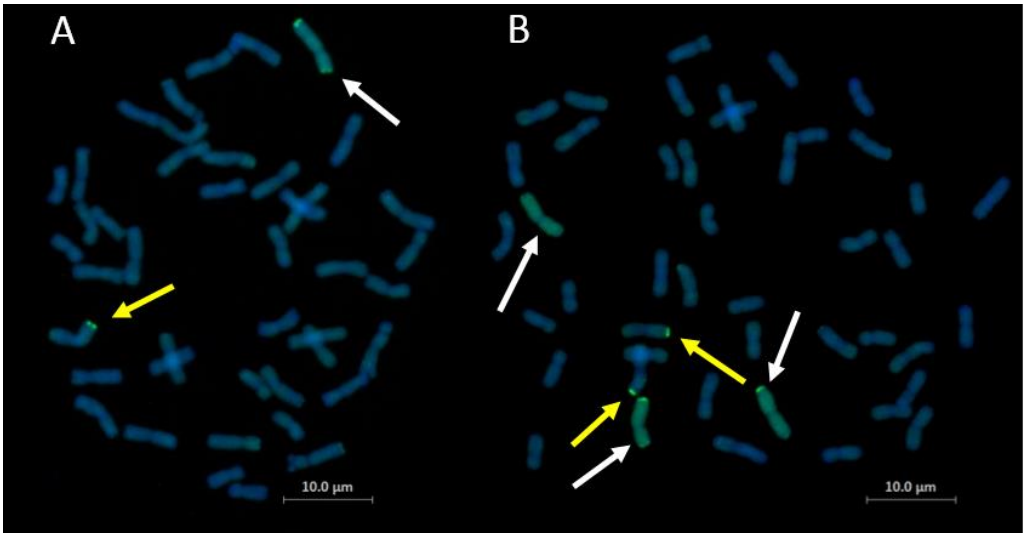


Figure VIII-3 Single-colour GISH image showing the genomic composition of two F_1 wheat/*Ae. sharonensis* addition lines

Ae. sharonensis is green (indicated by the white arrow), wheat is blue. The T4BS.4BL-4S^{sh}L translocated chromosome is indicated by the yellow arrow. A) Metaphase spread of a monosomic addition line with a single T4BS.4BL-4S^{sh}L translocated chromosome, B) Metaphase spread of a multisomic addition line with two T4BS.4BL-4S^{sh}L translocated chromosomes.

VIII.3.3.2. *Genomic composition of the BC_1 generation*

Single-colour GISH was carried out successfully on the fourteen BC_1 lines. Thirteen lines did not carry any chromosomes of *Ae. sharonensis* (**Figure VIII-4 A**) and one line carried one complete chromosome of *Ae. sharonensis* (**Figure VIII-4 B**). All lines contained forty-two chromosomes of wheat.

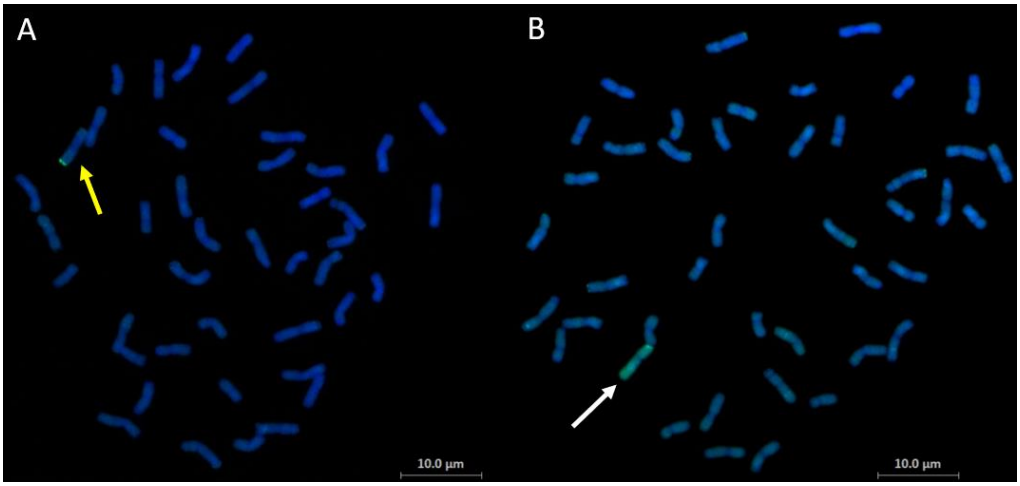


Figure VIII-4 Single-colour GISH image showing the genomic composition of two BC_1 lines

Ae. sharonensis, green (indicated by the white arrow), wheat, blue. The T4BS.4BL-4S^{sh}L translocated chromosome is indicated by the yellow arrow. A) Metaphase spread of a line with no *Ae. sharonensis* chromosomes and a single T4BS.4BL-4S^{sh}L translocated chromosome, B) Metaphase spread of a monosomic wheat/*Ae. sharonensis* addition line.

VIII.3.3.3. *Genomic composition of the F_2 generation*

Single-colour GISH was carried out successfully on thirty-six of the thirty-seven F_2 lines (**Table VIII-4**). Twenty-two lines carried between half a chromosome to three complete chromosomes of *Ae. sharonensis*. Seventeen lines carried a single chromosome of *Ae. sharonensis* and one line a single arm of a chromosome. One translocated chromosome was detected (**Figure VIII-5**). This chromosome was composed mostly by the chromatin of *Ae. sharonensis*.

Table VIII-4 Classification of the F_2 lines produced according to the number of wheat and *Ae. sharonensis* chromosomes

0.5 indicates the presence of a half chromosome, W/S^{sh} means a wheat/*Ae. sharonensis* translocated chromosome

Designation	Composition	No. of F_2 lines
Wheat	42W	14
Monosomic substitution	41W + 1S^{sh}	1
Telosomic addition	42W + 0.5S^{sh}	1
Monosomic addition	42W + 1S^{sh}	16
Multisomic addition	42W + (2-3)S^{sh}	3
Monosomic addition and translocated chromosome	42W + 1S^{sh} + W/S^{sh}	1

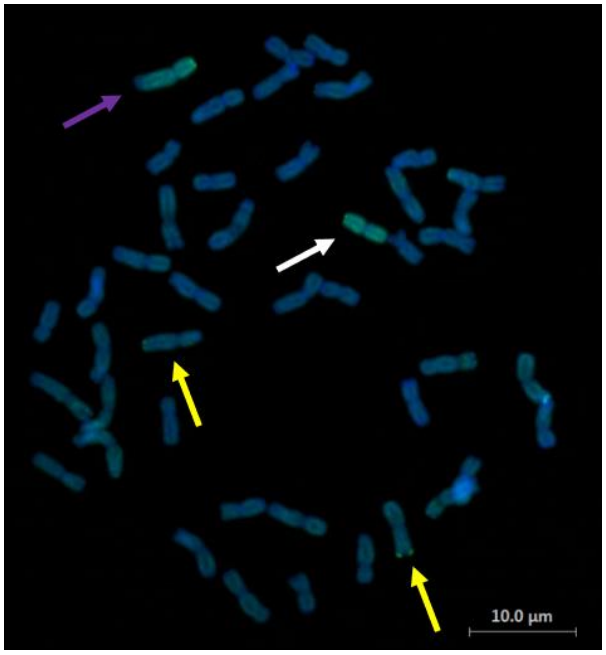


Figure VIII-5 Single-colour GISH image showing the genomic composition of one F_2 line

Metaphase spead of a line with one complete *Ae. sharonensis* chromosome (indicated by the white arrow), one wheat/*Ae. sharonensis* translocated chromosome (indicated by the purple arrow) and two T4BS.4BL-4S^{sh}L translocated chromosome (indicated by the yellow arrow). *Ae. sharonensis* - green, wheat - blue

VIII.3.4. Combination of the GISH and fertility results

Figure VIII-6 represents the fertility level of lines in relation to the presence or absence of chromosomes of *Ae. sharonensis* in the nucleus. The lines which did not carry any chromosomes of *Ae. sharonensis* were all sterile. The F_2 lines

which carried a range of the genome of *Ae. sharonensis* set 0 to 2.02 seeds/spikelet.

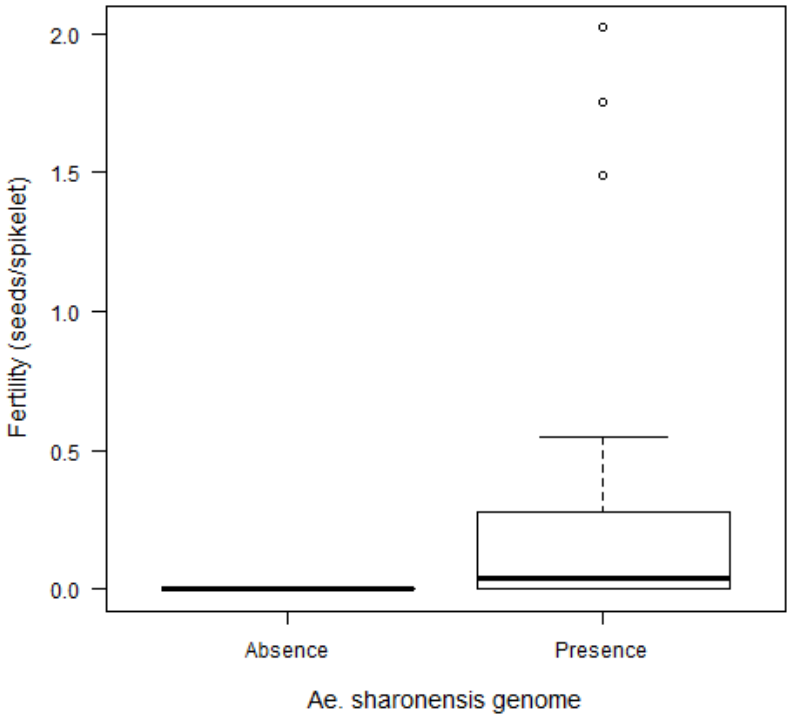


Figure VIII-6 Fertility level of the F_2 lines without or with *Ae. sharonensis* chromosomes in their genome

In the box plots, the boundary of the box closest to zero indicates the 25th percentile, a black line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles. Points above the whiskers indicate outliers outside the 90th percentiles.

VIII.3.5. Characterization of the fertile lines by KASP markers

Out of the 36 KASP markers tested, one did not amplify correctly or did not show any polymorphism between wheat and the *Ae. sharonensis* genome (**Table VIII-5**). A total of 35 polymorphic markers were therefore used which covered the complete *Ae. sharonensis* genome (**Table VIII-5**). Two markers initially located on linkage groups 4 and 6 were moved to linkage 5.

Table VIII-5 Number of KASP markers available, tested and amplified for each *Ae. sharonensis* linkage group

Linkage group	No. of polymorphic markers	No. of markers tested	No. of markers which amplified
1	4	4	4
2	15	7	7
3	6	4	4
4	10	7	7
5	9	5	5
6	7	6	5
7	3	3	3
Total	54	36	35

These KASP markers were run on the twelve fertile F_2 lines (**Figure VIII-7**). The results of the homozygous 6AL-6S^{sh}L.6S^{sh}S translocation line confirmed the position of five markers on linkage group 6, i.e. these markers showed a homozygous call for the allele of *Ae. sharonensis* as expected. The fertile lines were grouped by their pedigree to control the consistency of the results, i.e. line F_1.3 carried a single chromosome of *Ae sharonensis* so a unique lingkage group should be detected in the its progeny - lines F_2.3, F_2.7 and F_2.12, which was the case. Line F_2.6, (the only fertile progeny of F_1.1) carried linkage groups 4 and 6. The progeny of F_1.2 carried either linkage group 4 (F_2.1 and F_2.2) or linkage group 6 (F_2.8, F_2.10, F_2.11 and F_2.13) or both (F_2.5 and F_2.9). The progeny of F_1.3 carried linkage group 5.

Line genotyped		Fertile F2 lines												
F1 parent		F1.1	F1.2								F1.3			6S.6A
LG	Marker	6	1	2	5	8	9	10	11	13	7	12	3	
1	WRC0038	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0057	W	W	W	W	W	W	W	W	?	W	W	W	W
	WRC0095	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0127	W	W	W	W	W	W	W	W	W	W	W	W	W
2	WRC0159	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0219	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0225	W	?	W	W	W	W	W	W	W	W	W	W	W
	WRC0230	W	W	W	?	W	W	W	W	W	W	W	W	W
	WRC0236	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0277	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0280	W	W	W	?	W	W	W	W	W	W	W	W	W
3	WRC0367	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0379	W	W	W	W	W	W	W	W	?	W	W	W	W
	WRC0381	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0405	W	W	W	W	W	W	W	W	W	W	W	W	W
4	WRC0461	H	H	H	H	W	H	W	W	W	W	W	W	W
	WRC0466	H	H	H	H	W	H	W	W	W	W	W	W	W
	WRC0485	H	H	H	?	W	H	W	W	W	W	W	W	W
	WRC0514	H	H	H	?	W	H	W	W	W	W	W	W	W
	WRC0523	H	H	H	?	W	H	W	W	W	W	W	W	W
	WRC0551	H	H	H	H	W	H	W	W	W	W	W	W	W
5	WRC0614	W	W	W	W	W	W	W	W	W	H	H	H	W
	WRC0619	W	W	W	W	W	W	W	W	W	H	H	H	W
	WRC0625	W	W	W	W	W	W	W	W	W	H	H	H	W
	WRC0646	W	W	W	W	W	W	W	W	W	H	H	H	?
	WRC0471	W	W	W	?	W	W	W	W	W	H	H	H	W
	WRC0734	W	W	W	W	W	W	W	W	W	H	H	H	?
	WRC0661	W	W	W	W	W	W	W	W	W	W	W	W	W
6	WRC0717	H	W	W	H	H	H	H	H	H	W	W	W	S
	WRC0737	W	W	W	H	H	S	H	H	H	W	W	W	S
	WRC0777	S	W	W	H	H	S	H	H	H	W	W	W	S
	WRC0765	H	W	W	H	H	H	H	H	H	W	W	W	S
7	WRC0923	W	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0954	?	W	W	W	W	W	W	W	W	W	W	W	W
	WRC0965	W	W	W	W	W	W	W	W	W	W	W	W	W

Figure VIII-7 Graphical representation of the KASP genotyping of the fertile wheat/*Ae. sharonensis* lines and 6AL-6S^{sh}L.6S^{sh}S translocation line

Markers are ordered by linkage group and are scored as "W" for wheat allele (blue colour), "H" for heterozygous (red colour) and "S" for *Ae. sharonensis* allele (grey colour). Missing values are indicating by "?" (white colour). The numbers 1 to 13 indicate the F_2 code, the bold frame separates the progeny from the three different fertile F_1 lines (F_1.1, F_1.2 and F_1.3). "6S.6A" means the 6AL-6S^{sh}L.6S^{sh}S translocation line (Marais et al., 2006).

VIII.3.6. Combination of the fertility, GISH and KASP markers

Table VIII-6 summarizes the results of the characterization of the fertile F_2 lines and shows that the results obtained with the two different methods are consistent. For example, a complete chromosome of *Ae. sharonensis* was detected by GISH in line F_2.1 and the genotyping results indicated the presence of linkage group 4S^{sh}. In line F_2.5, the presence of two complete chromosomes of *Ae. sharonensis* were detected cytologically, chromosomes which were characterized by KASP markers as 4S^{sh} and 6S^{sh}. The presence of chromosome 4S^{sh} was associated with a fertility level of 0.02 seeds/spikelet. When chromosome 4S^{sh} was combined with chromosome 6S^{sh} the fertility ranged

from 0.14 to 0.52 seeds/spikelet. The lines which carried only chromosome $6S^{sh}$ set 0.28 to 2.02 seeds/spikelet. The presence of chromosome $5S^{sh}$ caused a restoration of fertility from 0.06 to 1.75 seeds/spikelet.

Table VIII-6 Comparison between GISH and KASP marker results of the fertile F_2 lines

F_2 line code	**Fertility (seeds/spikelet)**	**No. of *Ae. sharonensis* chromosomes (GISH)**	**Linkage group detected (KASP)**
F_2.1	0.02	1	$4S^{sh}$
F_2.2	0.02	1	$4S^{sh}$
F_2.3	0.06	1	$5S^{sh}$
F_2.5	0.14	2	$4S^{sh}$ + $6S^{sh}$
F_2.6	0.25	1+2*0.5	$4S^{sh}$ + $6S^{sh}$
F_2.7	0.28	1	$5S^{sh}$
F_2.8	0.28	1	$6S^{sh}$
F_2.9	0.52	3	$4S^{sh}$ + $6S^{sh}$
F_2.10	0.55	1	$6S^{sh}$
F_2.11	1.49	1	$6S^{sh}$
F_2.12	1.75	1	$5S^{sh}$
F_2.13	2.02	1	$6S^{sh}$

Information was lacking in a few cases to exactly identify the genomic composition of the line. For example, line F_2.6 carried one complete chromosome and two telosomic chromosomes of *Ae. sharonensis* (GISH analysis). The genotyping results indicated the presence of linkage groups 4 and 6. It was not possible to conclude which chromosome had broken at the centromere giving rise to the telosomic chromosomes. Line F_2.9 contained three chromosomes of *Ae. sharonensis*. The genotyping results indicated the presence of chromosomes $4S^{sh}$ and $6S^{sh}$. However, it was again not possible to conclude which chromosome was homozygous. F_2.9 was characterized by GISH as an addition line, i.e. all 42 chromosomes of wheat were present. Thus the

KASP markers showed a heterozygous call even with the presence of two copies of the *Ae. sharonensis* chromosomes as the wheat homoeologous chromosomes were present in the line as well.

VIII.4. Discussion

T. timopheevii cytoplasm is considered as the most promising for commercial hybrid wheat programmes. Several *Rf* genes have been found in related species such as rye, *Ae. umbellulata* and also in some wheat varieties (Bahl and Maan, 1973; Curtis and Lukaszewski, 1993; Kurauchi et al., 1977). In this thesis the ability of *S. anatolicum* (refer to Chapter 4) and *T. timopheevii* (refer to Chapter 7) to restore this cytoplasm has been demonstrated. However, a monogenic and total restoration has not been found. All the species belonging to the wheat gene pool are potential restorers against *T. timopheevii*. In particular, the species of the Sitopsis section of the genus *Aegilops* are of interest, as one member, *Ae. speltoides*, is considered as the donor of the cytoplasm of *T. timopheevii* (Mori et al., 1997). As a member of the Sitopsis section, *Ae. sharonensis* is a potential carrier of restorer genes against the cytoplasm of *T. timopheevii*. However, because of the presence of a strong *Gc* gene on chromosome $4S^{sh}$, this potential has not been investigated previously. The breaker element of the *Gc2* gene was knocked out and a wheat/*Ae. sharonensis* T4BS.4BL-$4S^{sh}$L translocation line with a mutated *Gc2* element was produced (Friebe et al., 2003), allowing the production of wheat/*Ae. sharonensis* introgression lines without chromosome $4S^{sh}$ (Marais et al., 2010; Millet et al., 2014). The aim of this chapter was the production of wheat/*Ae. sharonensis* introgression lines to test the ability of *Ae. sharonensis* to restore the cytoplasm of *T. timopheevii*.

<u>Production of wheat/*Ae. sharonensis* addition lines</u>

The crossing programme was based on the CS/*Gc* mutant line developed by Friebe et al., (2003) and was designed to remove chromosome $4S^{sh}$ and thus isolate different chromosomes of *Ae. sharonensis* in a wheat background. The genome of *Ae. sharonensis* was transferred via a wheat/*Ae. sharonensis*

amphidiploid (2n = 8x = 56, AABBDDS^{sh}S^{sh}). The amphidiploid was used as the female parent and the CS/*Gc* mutant line as male parent to increase the success of the crosses. It has been shown that the use of the higher ploidy level as the female, i.e. the amphidiploid, caused the highest frequency of shrivelled seeds (Thompson, 1930). This was confirmed in Chapters 3 and 5 of this thesis. A total of 55 crosses produced 74 F_1 seeds (②), all shrivelled. Less than 30% of the F_1 seeds sown germinated.

The shrivelled seeds and the low germination rate can be explained by the unbalanced genome of the embryo causing disruption in the production of the endosperm (Johnston et al., 1980) and thus the failure of germination (refer to Chapter I.4.2.2). For that reason, the embryos of these F_1 seeds were rescued and transferred to culture medium, which replaces the endosperm and provides nutrients to the developing embryo. In addition, it has been shown that the *Gc* chromosome 4S^{sh} causes chromosome damage in early embryo and endosperm development (King and Laurie, 1993). In comparison, in the absence of *Ae. sharonensis*, all the crosses carried out with the CMS lines and CS/*Gc* mutant were successful, no shrivelled seeds were produced and all the F_1 sown germinated successfullyy (①). The F_1 resulting from the crosses of the amphidiploid with the CS/*Gc* mutant (②) produced very low quantities of pollen which limited the pollination of the CMS. The low pollen production of F_1 (②) could again be the result of an unbalanced genome as a haploid genome of *Ae. sharonensis* is present causing reduced chromosome paring during meiosis (De Storme and Mason, 2014). A total of seventeen seeds were produced with only 18% shrivelled. This lower proportion is possibly due to the reduced number of *Ae. sharonensis* chromosomes, the absence of chromosome 4S^{sh} in some cases and the presence of the T4BS.4BL-4S^{sh}L translocated chromosome when the 4S^{sh} chromosome is present. Only five seeds (③) resulting from the pollination of the CMS-CS/*Gc* mutant by the amphidiploid-CS/*Gc* mutant, germinated and were characterized as addition lines with one to three chromosomes of *Ae. sharonensis* by single colour GISH.

The germination success of the F_2 and BC_1 seeds was above 60%, an increase which could be attributed to the higher stability of the genome and the elimination of the *Gc* chromosome. The cytological characterization of the F_2 and BC_1 lines highlighted the higher percentage of the complete elimination of the genome of *Ae. sharonensis* in the BC_1 (93%) compared to the F_2 (39%).

Seventeen monosomic addition, four multisomic and one monosomic substitution wheat/*Ae. sharonensis* lines were produced. A total of thirty-five KASP markers were used to characterize the chromosomes of *Ae. sharonensis*. Chromosomes $4S^{sh}$, $5S^{sh}$ and $6S^{sh}$ were detected. This result reflects the ability of the CS/*Gc* mutant line to remove chromosome $4S^{sh}$ and to produce monosomic wheat/*Ae. sharonensis* addition lines. The elimination of the gametocidal chromosome $4S^{sh}$ by using the same mutant line has been already reported in different studies (Millet et al., 2014, 2017). Until now, chromosome $4S^{sh}$ was always present in the background of the addition lines of the other chromosomes (Zhang et al., 2001). In this study monosomic addition lines with chromosomes $4S^{sh}$, $5S^{sh}$ and $6S^{sh}$ were produced. No addition lines involving chromosomes $1S^{sh}$, $2S^{sh}$, $3S^{sh}$ and $7S^{sh}$ were detected. This can be attributed to two different reasons. The first one is the very low number of seeds produced. Only five F_1 plants germinated (the plants coming from three different crosses). The second reason is the selection of the plants for their male fertility, discarding the plants non carrier of a restorer chromosome. Only the progeny of three of these F_1 plants were studied as the other two were male sterile. In addition, one translocated chromosome was detected in one F_2 line. This chromosome was composed by a large segment of *Ae. sharonensis* (chromosome $5S^{sh}$) and a very small distal segment of wheat. This translocation occurred spontaneously as the *Ph1* locus was present in the wheat background. Spontaneous crossover between *Ae. sharonensis* chromosomes and its homoeologous chromosomes from wheat can occur because of the high similarity between the genomes (Zhang et al., 2001). Spontaneous translocations have been reported involving chromosomes $4S^{sh}$ (Friebe et al., 2003) and $6S^{sh}$ (Marais et al., 2003).

Ability of *Ae. sharonensis* to restore *T. timopheevii* cytoplasm

Three of the F_1 lines (③) were male fertile setting an average of 0.5 seeds/spikelet (the control set 2.2 seeds/spikelet). At least one restorer factor was thus present in the male parent of these three lines (②). Three different sources were present in these parental lines: *Ae. sharonensis*, the CS/*Gc* mutant line and Chinese Spring used to create the wheat/*Ae. sharonensis* amphidiploid. The presence of chromosomes of *Ae. sharonensis* were detected in the three lines by GISH analysis (two lines carried two chromosomes of *Ae. sharonensis* and the third line a single chromosome of *Ae. sharonensis*).

The potential ability of Chinese Spring and the CS/*Gc* mutant to restore fertility was tested by pollinating the spring type CMS lines with these two genotypes. The progeny were male sterile, indicating that no restorer factors were present in the genome of Chinese Spring and the CS/*Gc* mutant (at least against the cytoplasm of *T. timopheevii* transferred to a spring wheat). These results again confirmed the results presented in Chapter 3 where Chinese Spring was shown to restore fertility only of the winter type CMS. The restoring (Kučera, 1982) and non-restoring (Ahmed et al., 2001) ability of Chinese Spring against *T. timopheevii* cytoplasm has been reported, highlighting the importance of the wheat background (Virmani and Edwards, 1983).

These results allow us to conclude that the genome of *Ae. sharonensis* carries one or several restorer factors against the cytoplasm of *T. timopheevii*. However, the level of restoration observed in the F_1 lines was very low with a high variability within the same plant, indicating a poor stability. The progeny of these three fertile F_1 were studied in order to individualize the chromosomes of *Ae. sharonensis*, to stabilize the genetic background, to enhance the level of fertility and to increase the size of the population studied. Back-crossed and selfed-seeds were germinated. Unfortunately, no BC_1 plants set selfed-seeds. The F_2 lines carried between zero to three chromosomes of *Ae. sharonensis* in addition to the forty-two chromosomes of wheat. The

fourteen lines which did not carry any chromosomes of *Ae. sharonensis* were all male sterile. This confirms that the genome of *Ae. sharonensis* carries a restorer factor against *T. timopheevii* cytoplasm. Thirteen F_2 addition lines were male fertile, with a fertility ranging from 0.02 to 2.02 seeds/spikelet. The presence of chromosomes $4S^{sh}$, $5S^{sh}$ and $6S^{sh}$ was detected by KASP markers. The highest fertility levels were observed in the lines carrying chromosomes $5S^{sh}$ (1.75 seeds/spikelet) and $6S^{sh}$ (1.49 and 2.02 seeds/spikelet). The presence of chromosome $4S^{sh}$ caused a very low restoration level with 0.02 seeds/spikelet.

No previous studies have reported the above results. Some parallels could be drawn with the restorer genes present on chromosomes 5D, 6B and 6D of wheat against *T. timopheevii* cytoplasm (Bahl and Maan, 1973, Yen et al., 1969). However, the expression of the fertility with *Ae. sharonensis* was not stable. For example, in some cases, a line with chromosome $5S^{sh}$ or $6S^{sh}$ expressed a very low level of restoration or was male sterile. This incomplete penetrance of the restorer factor has been reported in the case of *Rfc4* on chromosome 4R of rye (Curtis and Lukaszewski, 1993) and *Rf1* on chromosome 1A of wheat (Geyer et al., 2018). It has been shown that environment and genetic background influence the expression of the restoration (Virmani and Edwards, 1983). In this work, genetic reasons might have to be considered to explain the incomplete penetrance as the plants were grown in the same conditions. Modifier loci play an essential role in fertility restoration as they significantly influence expression of the restorer genes, which can be prevented in their absence (Robertson and Curtis, 1967). Another hypothesis is the DNA sequence elimination and epigenetic alteration caused by the hybrid genome (De Storme and Mason, 2014; Feldman and Levy, 2005; Tonosaki et al., 2016). Some $4S^{sh}$ and $6S^{sh}$ addition lines were fertile with a maximum fertility of 0.02 and 2.02 seeds/spikelet respectively. Three lines carried both chromosomes. The fertility level of these lines still ranged from 0.14 to 0.52 seeds/spikelet. The presence of chromosome $4S^{sh}$ with a functional *Gc* gene might also be the reason for the low fertility. Indeed,

through the generations, the T4BS.4BL-4S^{sh}L translocated chromosome disappeared.

Conclusion and perspectives

The genome of *Ae. sharonensis* has never been studied as a source of potential restorer genes against *T. timopheevii* cytoplasm because of the presence of a strong gametocidal gene on chromosome 4S^{sh} preventing the production of monosomic wheat/*Ae. sharonensis* addition lines and causing partial male fertility. A mutant copy of this *Gc* gene was successfully used in this programme and wheat/*Ae. sharonensis* 5S^{sh} and 6S^{sh} addition lines were developed. The material produced was characterized by KASP markers and GISH. We showed that chromosomes 5S^{sh} and 6S^{sh} cause a high level of restoration against the cytoplasm of *T. timopheevii*. These two restorer chromosomes seem to be a promising source for hybrid production.

Nevertheless, these are only preliminary results. Further investigations are needed to confirm them by studying a larger population of wheat/*Ae. sharonensis* 5S^{sh} and 6S^{sh} addition lines. In addition, the restorer factors of chromosomes 5S^{sh} and 6S^{sh} have to be tested in different wheat varieties to ensure their stability. In this study, total restoration was not observed. The fertility level, however, could possibly be increased by reducing the size of the *Ae. sharonensis* segment present in the line as despite a spontaneous wheat/*Ae. sharonensis* translocation being detected, the *Ae. sharonensis* segment still made up most of the chromosome. A strategy based on the use of the *ph1* mutant line was successfully developed to reduce the size of a segment of *Ae. sharonensis* (Marais et al., 2010, Millet et al., 2017). The production of restorer lines with a smaller segment of *Ae. sharonensis* genome will also allow a more precise localization of the restorer locus. The development of flanking markers will be needed to facilitate the tracking of the interesting segment through the generations. It will also be interesting to produce wheat/*Ae. sharonensis* 1S^{sh}, 2S^{sh}, 3S^{sh} and 7S^{sh} addition lines and to test their potential ability to restore the cytoplasm of *T. timopheevii*. The

wheat/*Ae. sharonensis* translocation lines developed could be used as potential sources for wheat improvement as chromosomes $5S^{sh}$ and $6S^{sh}$ of *Ae. sharonensis* are known to carry interesting rust resistance genes (Olivera et al., 2013; Yu et al., 2017).

IX. Chapter 9: Investigation of *Ae. sharonensis* cytoplasm as the base of a new CMS system

Summary

The aim of this chapter was the development of a new CMS system based on *Ae. sharonensis* cytoplasm. Wild *Ae. sharonensis* accessions were pollinated with the knocked-out mutant T4BS.4BL-4S^{sh}L translocation line in order to remove 4S^{sh} chromosome. Characterization of the lines was done by cytogenetics methods (GISH). Male sterile alloplasmic lines with 42 wheat chromosomes were produced. Some male fertile alloplasmic lines were detected suggesting that restorer genes were located on wheat genome. In parallel numerous wheat/*Ae. sharonensis* addition/substitution/translocation lines were produced. These lines are potential restorer lines against *Ae. sharonensis* cytoplasm.

IX.1. Introduction

IX.1.1. *Aegilops-Triticum* complex: a reservoir of potential cytoplasm causing male sterility in common wheat

Most of the efforts in hybrid wheat development have been focused on the cytoplasm of *T. timopheevii* as it causes total sterility and few deleterious effects (Wilson and Ross, 1962, Tsunewaki et al., 1976). However, no single restorer gene causing total and stable restoration has been introgressed into the wheat genome (Tsunewaki et al., 1976, 1996; Wilson and Driscoll, 1983). Species other than *T. timopheevii* are potential donors of a cytoplasm causing male sterility and thus the base of a new CMS system. A very precise analysis of the cytoplasms of *Aegilops* and *Triticum* species and their effects on wheat phenotype has been carried out (Tsunewaki, 2009; Tsunewaki et al., 1976, 1996, 2002). Hundreds of different hybrids have been produced using twelve common wheat nuclei and the cytoplasm of forty-seven *Triticum* and *Aegilops* species. Various morphological and physiological characters (i.e. growth

vigour, plant height, heading date or selfed seed fertility) were scored for each combination to classify the cytoplasm of the different species (**Table IX-1**).

Table IX-1 Effects of the cytoplasm of the *Aegilops* - *Triticum* complex on wheat phenotype (Tsunewaki, 2009; Tsunewaki et al., 1976, 1996, 2002)

Type	Species	Male sterility	Effect on the phenotype
A	*T. boeticum*	Severe sterility	Very severe negative
A^2	*T. mononoccocum*	Severe sterility	Very severe negative
B	*T. dicoccoides* *T. aestivum*	No sterility	No effect
C	*Ae. caudata*	Severe sterility	Slight negative
D	*Ae. cylindrica* *Ae. tauschii* *Ae. ventricosa*	No sterility	No effect
D^2	*Ae. crassa* *Ae. juvenalis* *Ae. vavilovii*	Light sterility	No effect
G	*T. araraticum* *T. timopheevii* *T. zhukovskyi*	Severe sterility	Slight negativ
M	*Ae. comosa*	Severe sterility	Severe negative
M^h	*Ae. heldreichii*	Severe sterility	Severe negative
M^o	*Ae. ovata*	Severe sterility	Severe negative
N	*Ae. uniaristata*	Moderate sterility	No effect
T	*Am. muticum*	Moderate sterility	Slight negative
S	*Ae speltoides*	Light sterility	No effect
S^b	*Ae. bicornis*	Light sterility	Slight negative
S^l	*Ae. sharonensis*	Severe sterility	Slight negative
S^{l2}	*Ae. longissima*	Severe sterility	Severe negative
S^v	*Ae. searsii* *Ae. kotschyi* *Ae. variabilis*	Moderate sterility	Slight negative
U	*Ae. umbellulata* *Ae. triuncialis* *Ae. biuncialis* *Ae. triaristata*	Severe sterility	Severe negative
U^2	*Ae. columnaris*	Severe sterility	Severe negative

A cytoplasm is a potential base of a CMS system if it causes stable male sterility and few deleterious effects on the wheat phenotype. For example, the cytoplasm of *Am. muticum* causes an extreme delay in heading (up to 20 days compared to wheat) which would be problematic for the commercial production of hybrid wheat. It has also been found that alloplasmic lines with *Am. muticum* cytoplasm showed supernumerary spikelets (Soltani et al., 2016). The B and D^2 types of cytoplasms (**Table IX-1**) showed high seed fertilities against all the nuclei tested. Similarly, the D, S and S^v cytoplasms (**Table IX-1**) expressed normal fertilities with most nuclei. None of the above cytoplasms induced total sterility with any of the tester nuclei. In contrast, the remaining cytoplasms caused total sterility against different tester nuclei.

In addition to the cytoplasm of *T. timopheevii*, some of the other cytoplasms from the *Aegilops-Triticum* complex have been studied as a potential base of a cytoplasmic male sterility system for hybrid wheat production: *Ae. kotschyi and Ae. variabilis* (Mukai and Tsunewaki, 1979), *Ae. umbellulata* and *Ae. caudata* (Tsunewaki, 1974), *Am. muticum* (Tsujimoto and Tsunewaki, 1984a). No total and durable fertility restorer genes were identified and translocated into the wheat genome. Few studies have reported the study of the *Ae. sharonensis* cytoplasm. It caused total male sterility with the Salmon, Spelta and Macha nuclei (Tsunewaki et al., 1976) and few deleterious effect on wheat cytoplasm (Nalepa, 1991). Nevertheless, genomic or genotyping studies did not have been carried out on these lines to confirm the elimination of the gametocidal chromosome.

IX.1.2. *Ae. sharonensis* and hybrid wheat

A cytoplasmic male sterility system is based on three lines which are the male sterile line, the maintenance line and the restorer line (refer to Chapter II.4.4.4). The production of the male sterile alloplasmic line (A-line) means that all the chromosomes of the female parent, the donor of the cytoplasm, have to been removed. In the case of the restorer line (R-line), chromosome engineering will be needed if the *Rf* gene is from a genome other than the

wheat genome (e.g. *Ae. sharonensis* genome). This *Rf* gene will have to be transferred into the wheat genome through the form of the smallest segment as possible to avoid linkage drag and to insure its stable transmission through the generations. However, the creation of the A-line and the R-line in the case of *Ae. sharonensis* is prevented by the presence of gametocidal (*Gc*) factors, specifically the factor on chromosome $4S^{sh}$ which showed the strongest effect (Endo, 1982, 1985; Finch et al., 1984b; McIntosh et al., 1998; Miller et al., 1982) (refer to Chapter I.7). By causing chromosome breakage in the gametes lacking it, this *Gc* chromosome will be transmitted in 98% of the progeny (King et al., 1991). A knock-out mutant of the breaker element of the *Gc* locus has been developed and transferred to Chinese Spring (Friebe et al., 2003) (refer to Chapter VIII.1.2.2.1).

IX.1.3. Aim and objectives of the chapter

The aim of this chapter was the study the potential of *Ae. sharonensis* as a base for a new CMS system:

- To produce a male sterile alloplasmic line (cytoplasm of *Ae sharonensis* and nucleus of common wheat)
- To produce various wheat/*Ae. sharonensis* introgression lines which could be potential restorer lines.

IX.2. Material and methods

IX.2.1. Plant material

Thirteen different accessions of *Ae. sharonensis* were used (584379, 584406, 584408, 584409, 584411, 584414 and TL02 obtained from the USDA; 2170007, 2170001, 2170004, 2170005, 2170006 and JIC18A500011 obtained from the Germplasm Resource Unit, JIC). Two wheat cultivars were used: the T4BS.4BL-$4S^{sh}$L translocation line (CS/*Gc* mutant) with the mutated allele of the *Gc* gene transferred to a Chinese Spring background (Friebe et al., 2003) and Paragon.

IX.2.2. Crossing programme

Figure IX-1 explains the crossing programme followed to produce wheat alloplasmic lines with the cytoplasm of *Ae. sharonensis* and wheat/*Ae. sharonensis* introgression lines. The thirteen different *Ae. sharonensis* accessions were pollinated by the CS/*Gc* mutant line, which was also used as the recurrent parent until the BC_2 generation. Paragon was used as the male parent to produce the BC_3 and BC_4 generations.

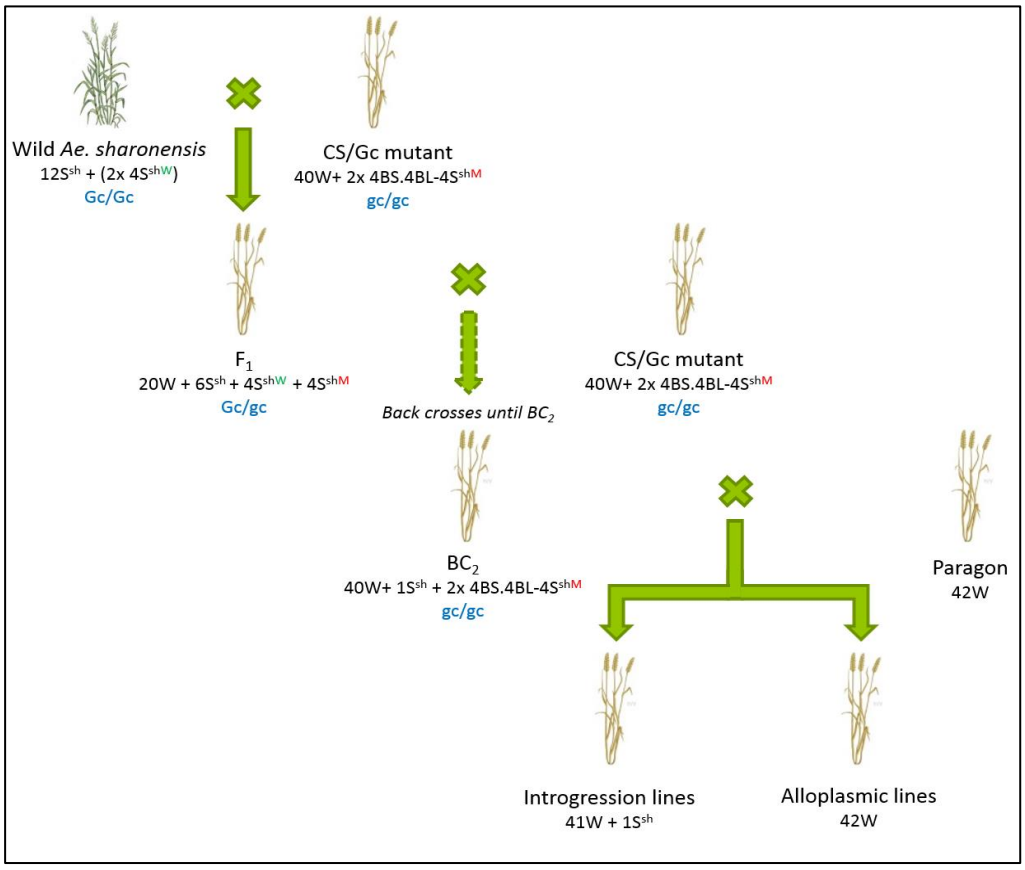


Figure IX-1 Crossing scheme for the production of wheat alloplasmic lines (*Ae. sharonensis)* and wheat/*Ae. sharonensis* introgression lines

$4S^{shW}$ means the wild allele (*Gc*) of the *Gc* gene on chromosome $4S^{sh}$ and $4S^{shM}$ means the mutated allele (*gc*) of the *Gc* gene (Friebe et al., 2003)

IX.2.3. Seed germination

Germination of the seeds was carried out according to the protocol (refer to Chapter II.1.1). As the F_1 seeds were shrivelled, embryo culture was used to enhance the germination success. The seeds were sterilised with 1 mL 5% ClNaO (10% solution-sigma Aldrich, Appendix 1), the embryo was rescued and placed on a culture medium (MS + 2% sucrose + 1% agarose, Appendix 1) under

sterile conditions. Once the seed germinated, it was sown into soil (John Innes compost No. 2). Wild *Ae. sharonensis* and the F_1 plants were put into vernalisation for eight weeks, the following generations and the wheats for four weeks (at 8°C with 12 hours light per day) and then transferred to the glasshouse (at 21°C, 16 hours light per day).

IX.2.4. Fertility restoration scoring

To score the male fertility restoration, one to three heads of the plants were bagged prior to anthesis and harvested after ripening. For each selfed spike, the number of seeds and the number of spikelets were counted and both were averaged by plant. The seed set of a plant was defined as the number of seeds divided by the number of spikelets. A plant without any selfed seed was considered as male sterile, whereas a plant with at least one seed was considered as male fertile. Common wheat was used as a control.

IX.2.5. Cytogenetic analysis

Preparation of metaphase spreads was described in Chapter II.3.3. Slides were probed as follows. Single colour GISH was carried out using total genomic DNA of common wheat as blocking DNA and total genomic DNA of *Ae. sharonensis* labelled by nick translation with Alexa Fluor 488-5-dUTP (Invitrogen; C11397) (green) as probe. The slides were then bleached for multicolour GISH and re-probed with total genomic DNA of *T. urartu* labelled by nick translation with Alexa Fluor 488-5-dUTP (Invitrogen; C11397) (green) and total genomic DNA of *Ae. tauschii* labelled with Alexa Fluor 594-5-dUTP (Invitrogen; C11400) (red). The total procedure was explained by Grewal et al. (2017). Total genomic DNA of *Ae. speltoides* was used as blocking DNA and the slides were counterstained with DAPI.

IX.3. Results

IX.3.1. Germplasm production

A total of 252 heads of *Ae. sharonensis* were pollinated with CS/*Gc* mutant giving a total of 427 F_1 seeds (1.7 seeds/crossed head). Seed set varied

according to the *Ae. sharonensis* accession used as maternal parent (**Table IX-2**).

Table IX-2 Number of F_1 seeds produced and germinated for each accession of *Ae. sharonensis*

Accession	Crosses made	F_1 seeds	F_1 seeds/Cross	F_1 seeds sown	F_1 seeds germinated	Germination rate
584379	10	1	0.10	0	-	-
584406	15	27	1.80	14	3	21.43%
584408	17	40	2.35	23	3	13.04%
584409	13	21	1.62	13	0	0%
584411	15	26	1.73	15	0	0%
584414	30	87	2.90	55	0	0%
2170007	14	0	0	0	-	-
2170001	24	77	3.21	38	3	7.89%
2170004	16	9	0.56	5	0	0%
2170005	16	3	0.19	0	-	-
2170006	21	38	1.81	22	0	0%
JIC18A500011	19	56	2.90	23	3	13.04%
TL02	42	42	1.00	25	0	0%

A total of 233 F_1 seeds were sown but only twelve germinated after embryo rescue (5.15%) (**Table IX-2**). These F_1 seeds were from accessions 584406, 584408, 2170001 and JIC18A500011 and had a germination rate of 21.43%, 13.04%, 7.89% and 13.04% respectively. The back cross programme was carried out with these twelve F_1 plants.

IX.3.1.1. *Accession 584406*

The results of the crosses with accession 584406 are shown in **Table IX-3**. Two BC_1 seeds were produced from the pollination of fourteen heads of the three F_1 plants. These two seeds were shrivelled and did not germinate.

Table IX-3 Crosses results with the accession 584406

	F_1	**BC_1**
Seeds sown	13	2
Germination rate	21.43%	0%
Crosses made	14	-
Cross fertility	14.13%	-
Crossed seeds	2	-
Seeds/cross	0.14	-

IX.3.1.2. *Accession 584408*

The results of the crosses with accession 584408 are shown in **Table IX-4**. Seven BC_1 seeds were produced from the 45 F_1 heads pollinated. Five BC_1 seeds were sown and all of them germinated without rescuing the embryo. Crosses were carried on until the BC_5 generation. Germination rate, cross fertility and number of seeds/cross increased through the generations. Success of the crosses of the BC_4 dropped to 2.5 seeds/head crossed (compared to 10.48 seeds in the BC_3 generation) because of a high population of thrips in the glasshouse at this time.

Table IX-4 Crosses results with the accession 584408

	F_1	BC_1	BC_2	BC_2F_1	BC_3	BC_4
Seeds sown	13	5	19	24	34	4
Germination rate	13.04%	100%	63.16%	75%	85.29%	100%
Crosses made	45	20	44	-	26	4
Cross fertility	16.21%	55.42%	64.23%	-	86.17%	50%
Crossed seeds	7	21	28	-	304	10
Seeds/cross	0.16	1.05	3.52	-	10.48	2.5
Selfed	-	0	200		1058	19

IX.3.1.3. *Accession 2170001*

The results of the crosses with accession 2170001 are shown in

Table IX-5. Seven BC_1 seeds were produced from the 44 F_1 heads pollinated. Five BC_1 seeds were sown and all of them germinated without rescuing the embryo. Crosses were carried on until the BC_5 generation. Germination rate, cross fertility and number of seeds/cross increased through the generations.

Table IX-5 Crosses results with the accession 2170001

	F_1	BC_1	BC_2	BC_2F_1	BC_3	BC_4
Seeds sown	38	5	11	19	51	11
Germination rate	7.89%	100%	90.90%	100%	78.43%	100%
Crosses made	44	7	39	-	30	11
Cross fertility	16.02%	57.44%	72.05%	-	83.33%	81.22%
Crossed seeds	7	15	120	-	276	91
Seeds/cross	0.16	2.14	3.01	-	9.2	8.27
Selfed seeds	-	4	209		1974	454

IX.3.1.4. *Accession JIC18A500011*

The results of the crosses with accession JIC18A500011 are shown in **Table IX-6**. Two BC_1 seeds were produced from the pollination of eight heads of the three F_1 plants. These three seeds were shrivelled and did not germinate.

Table IX-6 Crosses results with the accession JIC18A500011

	F_1	**BC_1**
Seeds sown	23	2
Germination rate	13.04%	0%
Crosses made	8	-
Cross fertility	38.17%	-
Crossed seeds	3	-
Seeds/cross	0.38	-

IX.3.1.5. *Seeds shape and lines morphology*

Figure IX-2 shows the shape of the seeds for each generation compared to the two parents, *Ae. sharonensis* and Chinese Spring. *Ae. sharonensis* seeds showed a long and narrow shape. F_1 seeds were mostly shrivelled, narrower and flatter than the *Ae. sharonensis* seed. Through the back cross generations, the seeds became bigger and rounder. BC_4 and BC_5 seeds had a similar shape to the Chinese Spring seeds.

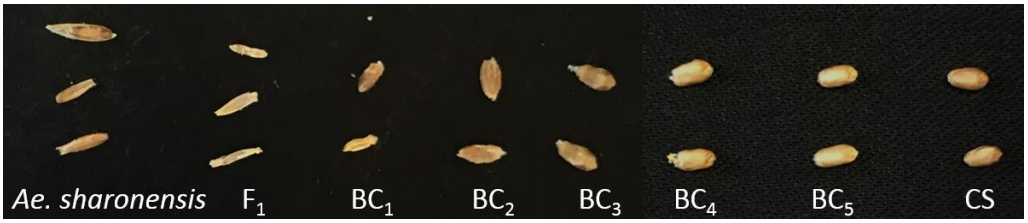


Figure IX-2 Seed shapes of *Ae. sharonensis*, the different generations of the *Ae. sharonensis* x wheat crosses and Chinese Spring (CS)

A similar pattern of results are shown in **Figure IX-3** which shows the phenotype and the head shape of the lines produced in comparison to the wild *Ae. sharonensis* accession. *Ae. sharonensis* morphology is characterized by a flattened and narrow spike with an awn and a thin, hairy leaf. Through the back cross generations, the lines showed broader and more glabrous leaves, taller stem and broader spikes with a higher number of spikelets.

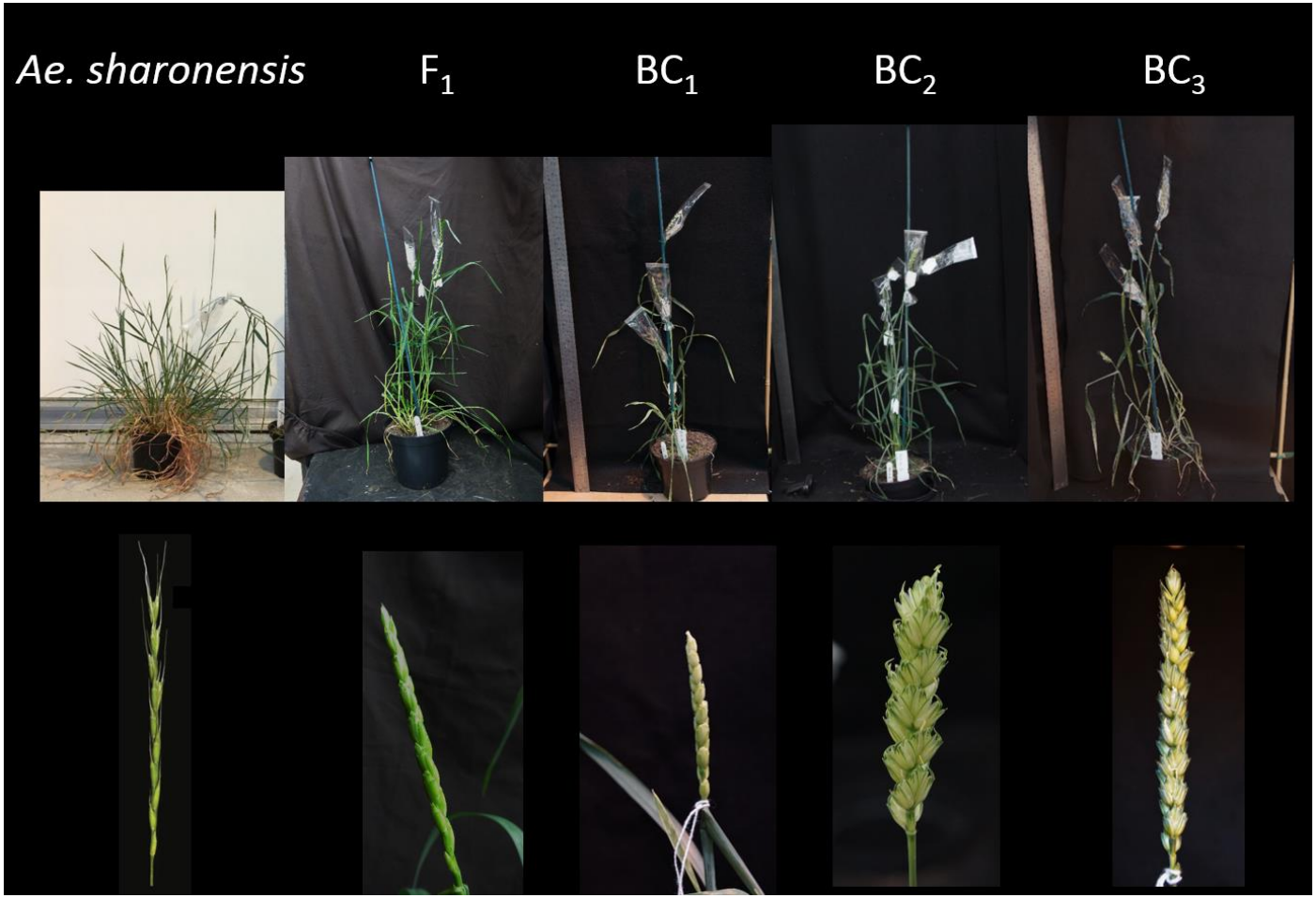


Figure IX-3 Phenotype and head shape of *Ae. sharonensis* and the different generations of the *Ae. sharonensis* x wheat crosses

IX.3.2. Characterization of the lines produced by GISH

Genomic composition of the lines produced was determined by carrying out GISH analysis.

IX.3.2.1. *Genomic composition of the parental lines*

Figure IX-4 shows a metaphase spread of *Ae. sharonensis* and the T4BS.4BL-4S^{sh}L translocation line (CS/*Gc* mutant) used as initial parents. Fourteen chromosomes were counted in the *Ae. sharonensis* accession. The CS/Gc mutant spread (**Figure IX-4 B**) shows the presence of the homozygous translocated chromosomes, T4BS.4BL-4S^{sh}L, with the fragment of *Ae. sharonensis* at the distal end of the wheat chromosome.

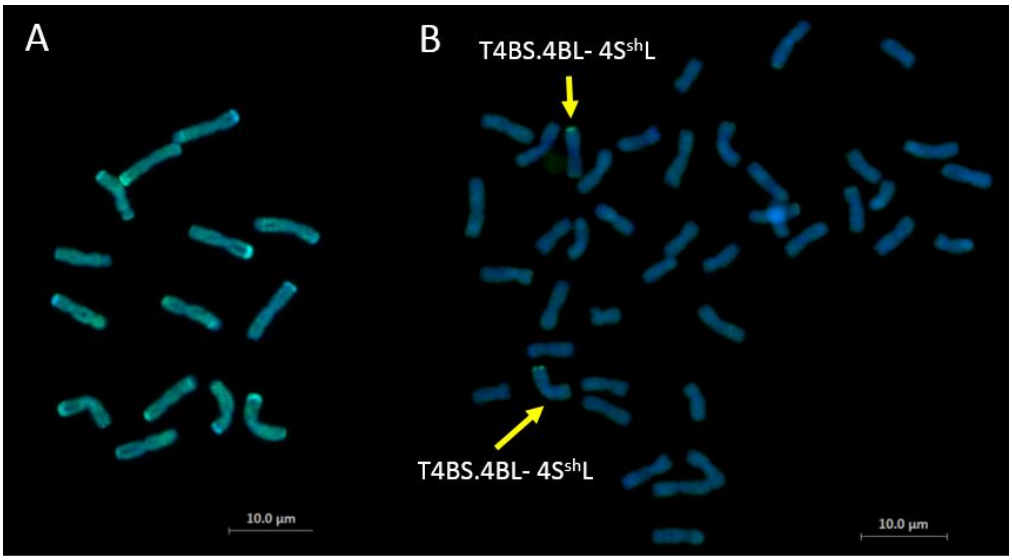


Figure IX-4 Single-colour GISH image showing the genomic composition of the parents

Ae. sharonensis-green, wheat – blue. A) *Ae. sharonensis* B) T4BS.4BL-4S^{sh}L translocation line (CS/*Gc* mutant). The T4BS.4BL-4S^{sh}L translocated chromosome is indicated by the yellow arrow.

IX.3.2.2. *Genomic composition of the F_1 generation*

Single-colour GISH was carried out on four F_1 lines (**Figure IX-5**). All contained twenty-one wheat chromosomes and seven *Ae. sharonensis* chromosomes, i.e. the total haploid chromosomes set of each parental genome. The translocated chromosome T4BS.4BL-4S^{sh}L was heterozygous.

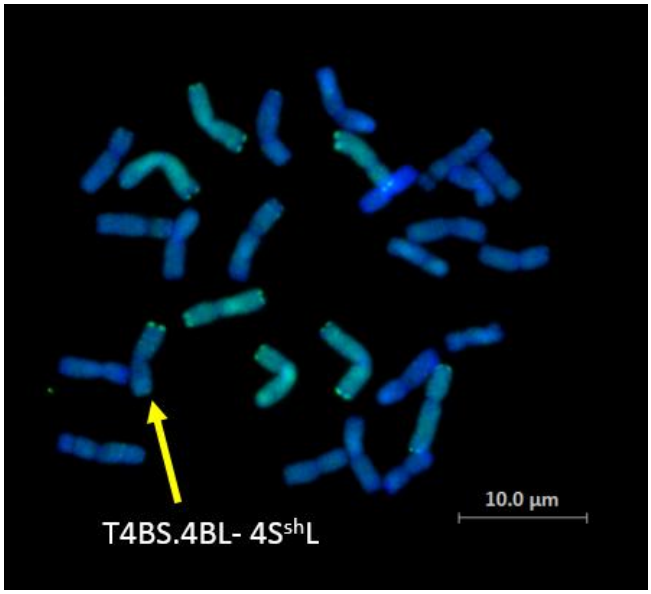


Figure IX-5 Single-colour GISH image showing the genomic composition of one F_1 line

Ae. sharonensis - green, wheat – blue. The T4BS.4BL-4S^{sh}L translocated chromosome is indicated by the yellow arrow.

IX.3.2.3. *Genomic composition of the BC_1 generation*

Single-colour GISH could only be carried out on eight BC_1 lines due to the quality of the metaphase spread. The number of wheat and *Ae. sharonensis* chromosomes were counted in the different lines (**Table IX-7**). The number of wheat chromosomes ranged from 36 to 43 and the number of *Ae. sharonensis* chromosomes from 5 to 7. The total chromosome number ranged from 41 to 49.

Table IX-7 Genomic composition of the BC_1 lines

Code	Accession	No. of wheat chromosomes	No. of *Ae. sharonensis* chromosomes	Total
BC_1.1	584408	37	7	44
BC_1.2	584408	41	6	47
BC_1.3	584408	43	6	49
BC_1.4	584408	40	7	47
BC_1.5	2170001	39	5	44
BC_1.6	2170001	42	7	49
BC_1.7	2170001	36	5	41
BC_1.8	2170001	42	6	48

IX.3.2.4. *Genomic composition of the BC_2 generation*

GISH was successfully carried out on all 22 BC_2 lines produced. The lines carried between 0 to 6 chromosomes of *Ae. sharonensis* (**Table IX-8**) and included two alloplasmic lines composed of a complete set of 42 wheat chromosomes (**Figure IX-6 A** and **B**), one monosomic and seven multisomic addition lines and six monosomic and five multisomic substitution lines (**Figure IX-6 C** and **D**). Five translocated wheat/*Ae. sharonensis* chromosomes were detected in these lines (**Figure IX-6 C** and **D**). The T4BS.4BL-4S^{sh}L translocated chromosome was homozygous in all the lines.

Table IX-8 Classification of the BC_2 lines produced according to their number of wheat and *Ae. sharonensis* chromosomes (W/S^{sh} means a wheat/*Ae. sharonensis* translocated chromosome)

Designation	**Composition**	**No. of BC_2 lines (584408)**	**No. of BC_2 lines (2170001)**
Monosomic substitution	$40W+ 1S^{sh}$	1	0
Monosomic substitution	$41W + 1S^{sh}$	3	1
Multisomic substitution	$40W + 2S^{sh}$ $39W + 3S^{sh}$	2	0
Monosomic addition	$42W + 1S^{sh}$	1	0
Multisomic addition	$42W + (2\text{-}6)S^{sh}$	4	3
Translocation	$40W + W/S^{sh}+ W/S^{sh}$	0	1
Addition and translocation	$(40\text{-}42)W + (1\text{-}3) + W/S^{sh}$	1	3
Alloplasmic	42W	0	2

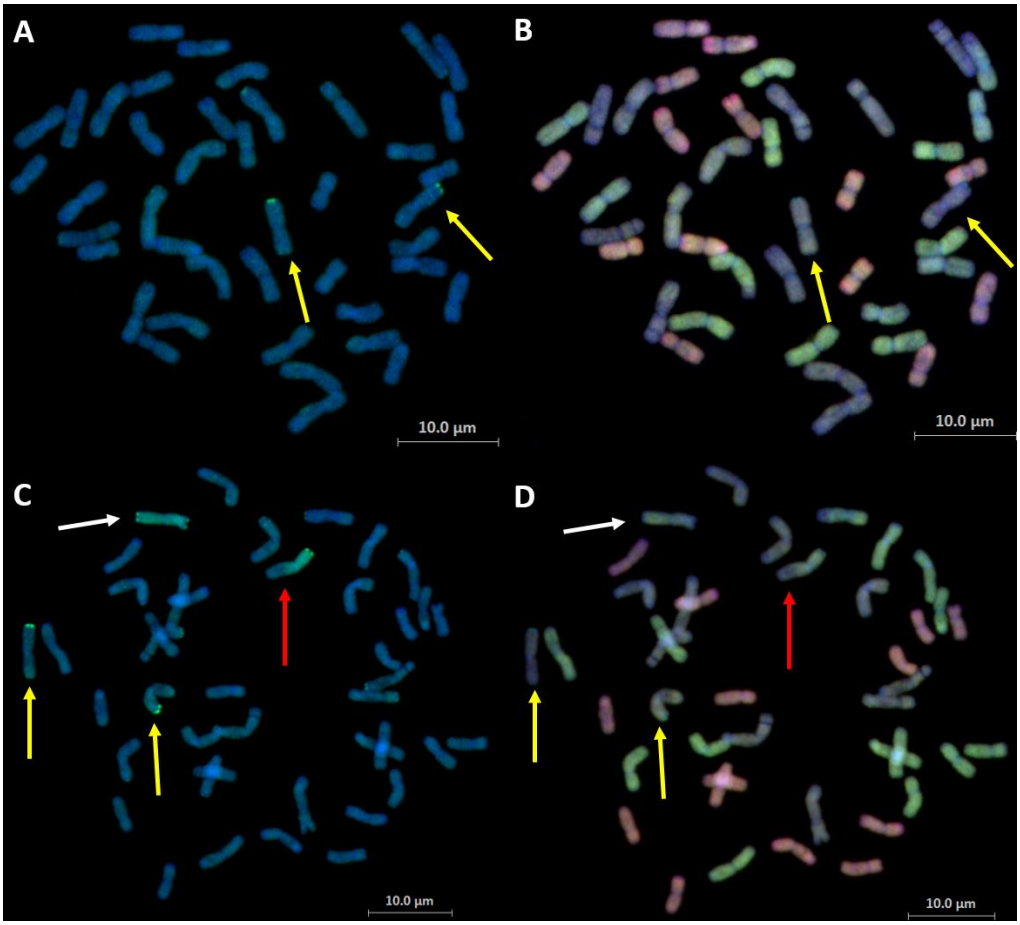


Figure IX-6 GISH images showing the genomic composition of BC_2 lines
A) Shows a spread of an alloplasmic line with *Ae. sharonensi*s (green) and wheat (blue). The T4BS.4BL-4S^{sh}L translocated chromosome is indicated by the yellow arrow. B) The same spread as A) with multi-colour GISH with A genome (green), B genome (blue) and D genome (red). C) Shows a spread of a line with a complete chromosome of *Ae. sharonensis* (white arrow) and a wheat/*Ae. sharonensis* translocated chromosome (red arrow) with *Ae. sharonensi*s (green) and wheat (blue), D) The same spread as C) showing a *Ae. sharonensis* chromosome recombined with the B genome of wheat with multi-colour GISH with the A genome (green), B genome (blue) and D genome (red)

IX.3.2.5. *Genomic composition of the BC_2F_1 generation*

From the 38 BC_2F_1 seeds germinated, GISH was successfully carried out on 37 lines. The lines carried between 0 to 4 chromosomes of *Ae. sharonensis* (**Table IX-9**) including 21 alloplasmic lines (7 with the cytoplasm of accession 584408 and 14 with the cytoplasm of accession 2170001), six lines carrying a single chromosome of *Ae. sharonensis* (2 with the cytoplasm of accession 584408 and 4 with the cytoplasm of accession 2170001) and five lines carrying two chromosomes of *Ae. sharonensis*. However, the BC_2 parent of four of these five

carried only a single chromosome of *Ae. sharonensis* leading to the conclusion that these four BC_2F_1 lines carried two copies of the same chromosome of *Ae. sharonensis*. Likewise, the one line with four chromosomes of *Ae. sharonensis* carried two copies of two chromosomes. Three lines carried a homozygous wheat/*Ae. sharonensis* translocated chromosome.

Table IX-9 Composition of the BC_2F_1 lines produced

***Ae. sharonensis* chromosome**	**No. of BC_2F_1 lines (584408)**	**No. of BC_2F_1 lines (2170001)**
0 chromosome	7	14
1 chromosome	4	2
1 chromosome + 1 translocated chromosome	0	2
1 chromosome + 1 translocated homozyogus chromosome	0	1
1 complete + 1 telosomic chromosome	1	0
2 chromosomes (homozyogous)	5 (4)	0
4 chromosomes (homozyogous)	1 (1)	0

IX.3.2.6. *Genomic composition of the BC_3 generation*

From the 69 BC_3 lines studied, GISH was successfully carried out on 65 lines. The lines carried between 0 to 2 chromosomes of *Ae. sharonensis* (

Table IX-10). The lines included a total of 40 alloplasmic lines (15 with the cytoplasm of accession 584408 and 25 with the cytoplasm of accession 2170001) with a wheat chromosome number ranging from 41 to 43, ten monosomic substitution lines and six monosomic and three disomic addition lines. Three lines with the cytoplasm of accession 2170001 carried a wheat/*Ae. sharonensis* translocated chromosome with the number of wheat chromosomes ranging from 40 to 42 (**Figure IX-7 A** and **B**).

Table IX-10. Classification of the BC_3 lines produced according to their number of wheat and *Ae. sharonensis* chromosomes (W/S^{sh} means a wheat/*Ae. sharonensis* translocated chromosome)

Designation	Composition	No. of BC_3 lines (584408)	No. of BC_3 lines (2170001)
Monosomic substitution	40W+ $1S^{sh}$	2	1
Monosomic substitution	41W + $1S^{sh}$	6	1
Monosomic addition	42W + $1S^{sh}$	2	4
Multisomic addition	42W + $2S^{sh}$	2	1
Translocation	40W + W/S^{sh} 41W + W/S^{sh} 42W + W/S^{sh}		1 1 1
Addition and translocation	41W + $1S^{sh}$ +1 W/S^{sh} 42W +$1S^{sh}$ + 1 W/S^{sh}	1	1 1
Alloplasmic	41W 42W 43W	5 8 2	3 22

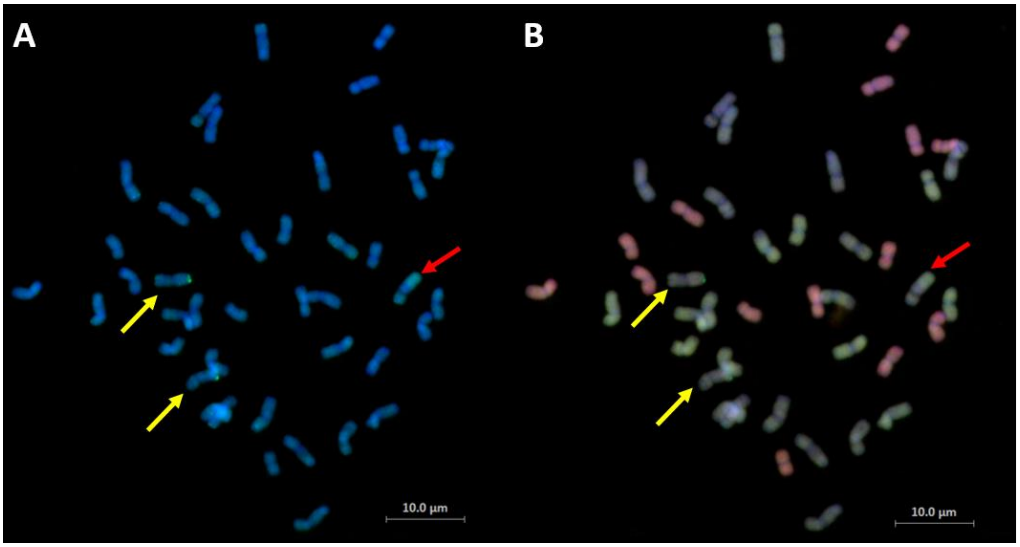


Figure IX-7 GISH images showing the genomic composition of one BC_3 line A). A metaphase spread of a line with a wheat/*Ae. sharonensis* translocated chromosome (red arrow) with *Ae. sharonensis* (green) and wheat (blue). B). Multi-colour GISH of the same spread as A) showing that the *Ae. sharonensis* chromosome recombined with the B genome of wheat (red arrow) with the A genome in green, B genome in blue and D genome in red. The line is composed of 14 A-chromosomes, 15 B-chromosomes and 14 D-chromosomes. The T4BS.4BL-$4S^{sh}$L translocated chromosome is indicated by the yellow arrow.

IX.3.2.7. *Genomic composition of the BC_4 generation*

GISH was successfully carried out on the 15 BC_4 lines sown all of which were alloplasmic. Six lines had lost the T4BS.4BL-4S^{sh}L translocated chromosome Four of the six had one wheat chromosome missing whereas the other two had a total set of wheat chromosomes (**Table IX-11** and **Figure IX-8).**

Table IX-11 Genomic composition of the alloplasmic BC_4 lines

Composition	No. of BC_4 lines (584408)	No. of BC_4 lines (2170001)
40W + T4BS.4BL-4S^{sh}L	2	1
41W + T4BS.4BL-4S^{sh}L	0	6
41W	2	2
42W	0	2

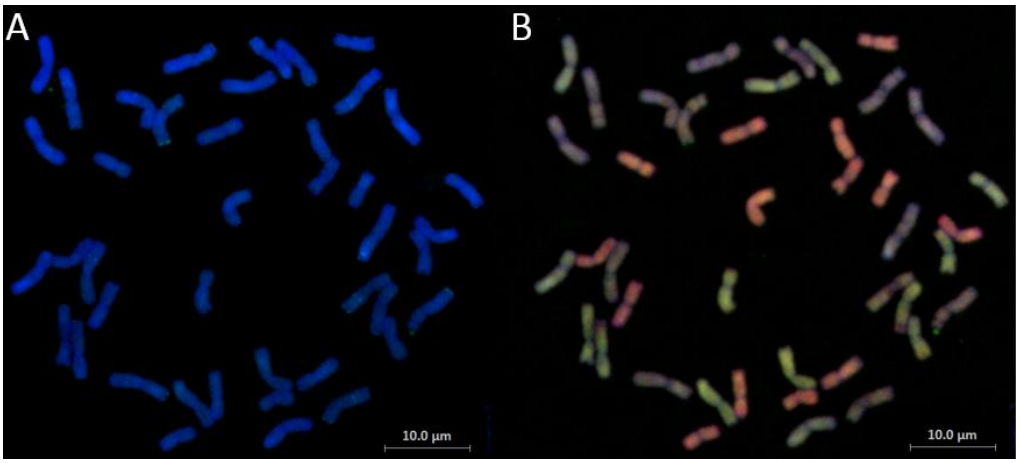


Figure IX-8 GISH images showing the genomic composition of one alloplasmic BC_4 line without the T4BS.4BL-4S^{sh}L translocated chromosome

A). Metaphase spread of an alloplasmic line with wheat in blue. B). Multi-colour GISH of the same spread as A) with the A genome in green (14 chromosomes), B genome in blue (14 chromosomes) and D genome in red (14 chromosomes).

IX.3.2.8. *Transmission rate of the Ae. sharonensis genome*

A complete chromosome of *Ae. sharonensis* was transmitted from the BC_2 to the BC_3 and BC_2F_1 generation in 44% of the back crossed offspring and in 50% of the selfed-progeny (**Figure IX-9**), wheras for a wheat/*Ae. sharonensis* translocated chromosome the transmission rate was 28% and 39% respectively.

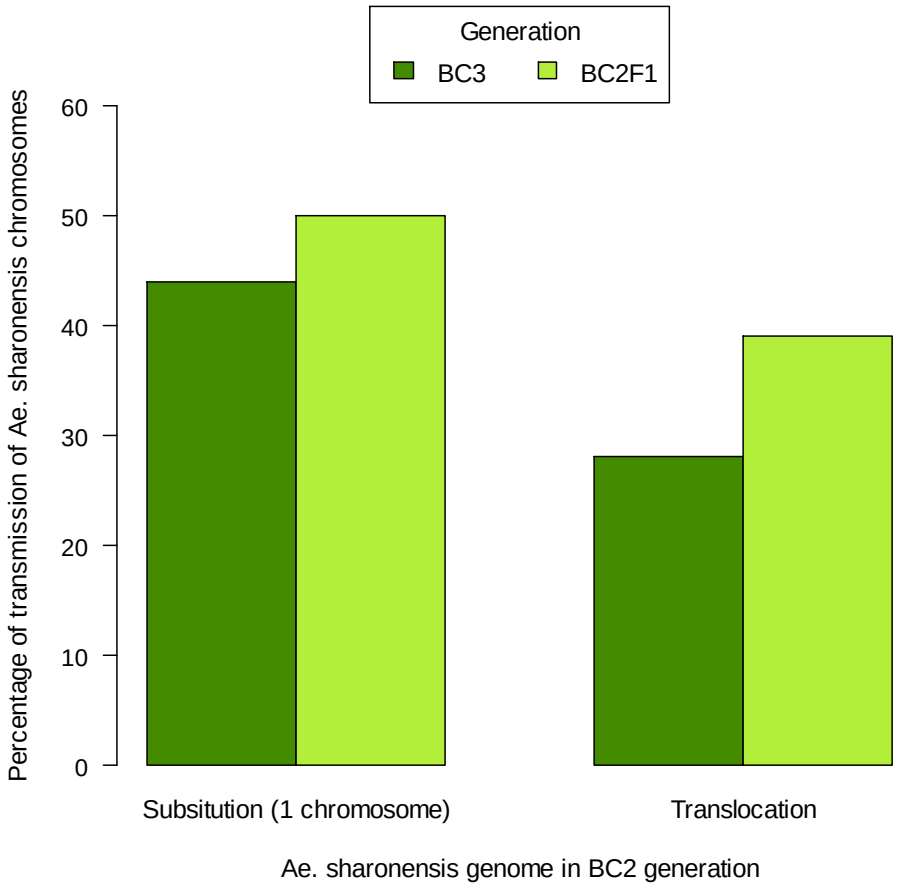


Figure IX-9 Transmission rate of *Ae. sharonensis* chromosomes

IX.3.3. Fertility scoring

All the F_1 lines grown were sterile. In the BC_1 generation only one line set some selfed-seeds, with an average of 0.33 seeds/spikelet. This line carried forty-two chromosomes of wheat and seven chromosomes of *Ae. sharonensis*. The fertility of the 22 BC_2 lines ranged from 0 to 2.07 seeds/spikelet (**Figure IX-10 A**). The control set 2.3 seeds/spikelet. High variation of fertility was observed within the individuals, especially for lines 15, 18, 19 and 20. Four lines were male sterile (lines 1, 2, 3 and 4). These lines carried between one and six chromosomes of *Ae. sharonensis*. Lines 7 and 20 did not carry any chromosomes of *Ae. sharonensis* and set 0.27 and 1.68 seeds/spikelet respectively. The fertility of the BC_2F_1 lines ranged from 0 to 2.62 seeds/spikelet (**Figure IX-10 B**). The control set 2.5 seeds/spikelet. A high variation of fertility was observed within the individuals, especially for lines 27, 31 and 32. Lines 1, 2, and 3 were male sterile. These lines carried a complete

chromosome of *Ae. sharonensis* and a homozygous wheat/*Ae. sharonensis* translocated chromosome, no *Ae. sharonensis* chromosome and four chromosomes of *Ae. sharonensis* respectively. The twenty alloplasmic lines set between 0 to 2.62 seeds/spikelet. The fertility of the 69 BC_3 lines ranged from 0 to 2.32 seeds/spikelet (**Figure IX-10 C**). The control set 3.2 seeds/spikelet. A high variation of fertility was observed within the fertile individuals, especially lines 49, 52, 54 and 55. A total of twelve lines were sterile (Lines 1 to 12). Five of these sterile lines did not carry any chromosomes of *Ae. sharonensis*, four carried a complete chromosome of *Ae. sharonensis* and three of them carried a complete chromosome of *Ae. sharonensis* as well as a translocated wheat/*Ae. sharonensis* chromosome. Thirty-five alloplasmic lines set selfed-seeds (the fertility ranged from 0.02 to 2.32 seeds/spikelet). The other fertile lines carried either a translocated chromosome (lines 34, 37 and 48), one chromosome of *Ae. sharonensis* (lines 20, 26, 31, 33, 41, 42, 46, 50, 53, 58, 59 and 60) or two chromosomes of *Ae. sharonensis* (lines 21, 30 and 62). The fertility of the BC_4 lines ranged from 0 to 2.13 seeds/spikelet (**Figure IX-10 D**). The control set 2.8 seeds/spikelet. Two lines were male sterile and had lost the T4BS.4BL-4S^{sh}L translocated chromosome. A high variation of fertility was observed within individuals, especially for lines 9, 10 and 14.

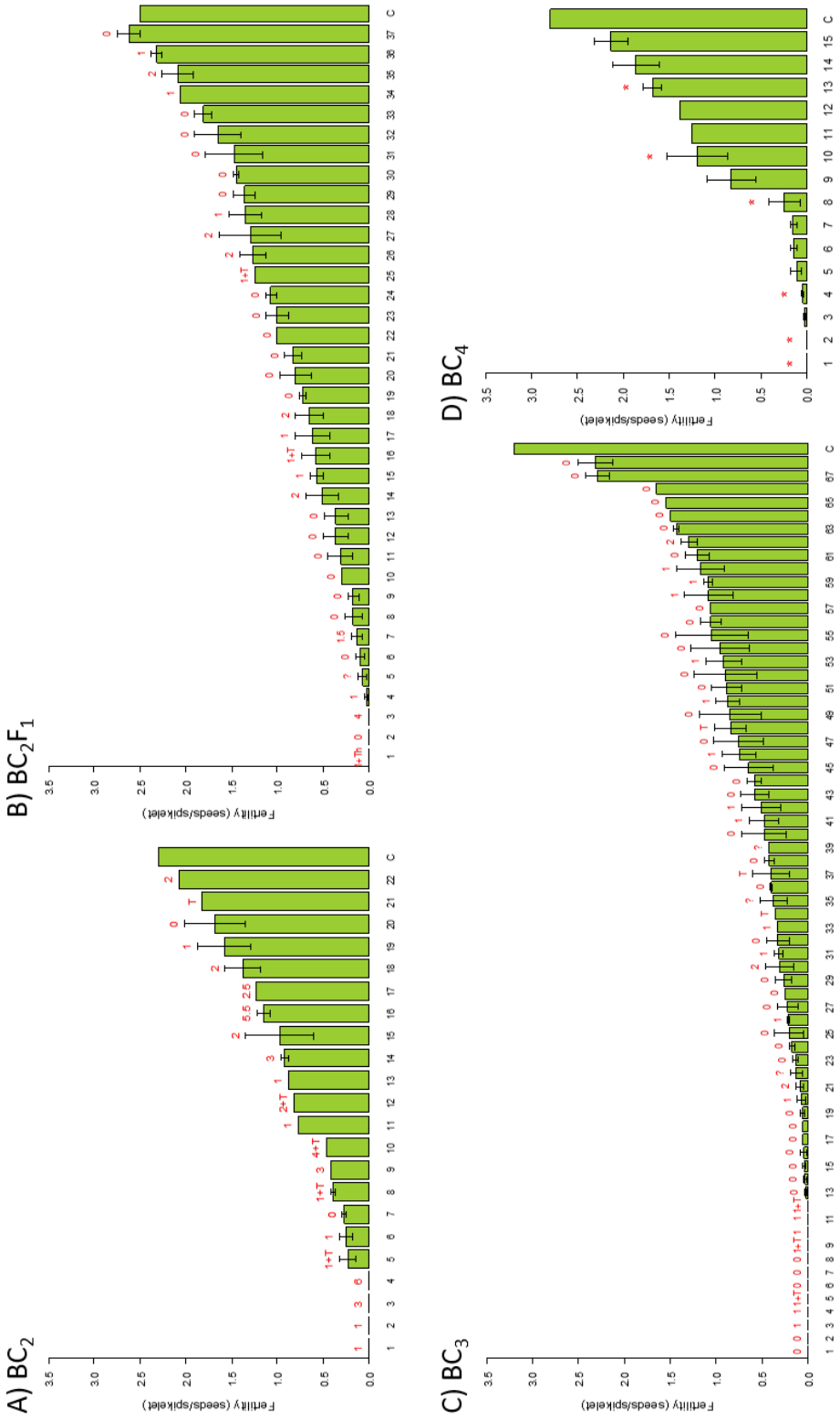


Figure IX-10 Distribution of the self-fertility level of the A) BC_2 lines, B) BC_2F_1 lines, C) BC_3 and D) BC_4

The control is designated by C (the bar on the right hand side). The number of *Ae. sharonensis* chromosomes present in the line is indicated in red above the plot, T means a wheat/*Ae. sharonensis* translocated chromosome, Th means a homozygous wheat/*Ae. sharonensis* translocated chromosomes. For the BC_4 lines the absence of the T4BS.4BL-4S^{sh}L translocated chromosome is indicated with the red *.

Figure IX-11 showes the evolution of the fertility through the generations. The F_1 and BC_1 lines were sterile (except one BC_1 line setting 0.3 seeds/spikelet). Self- fertility appeared at the BC_2 generation ranging from 0 to 2.07 seeds/spikelet with a median of 0.80 seeds/spikelet. BC_2F_1 lines showed a similar fertility pattern to the BC_2 generation. A decrease of the median was observed in the BC_3 and the BC_4 generations (0.40 and 0.20 seeds/spikelet respectively). The fertilities scored in the BC_4 lines were the most variable.

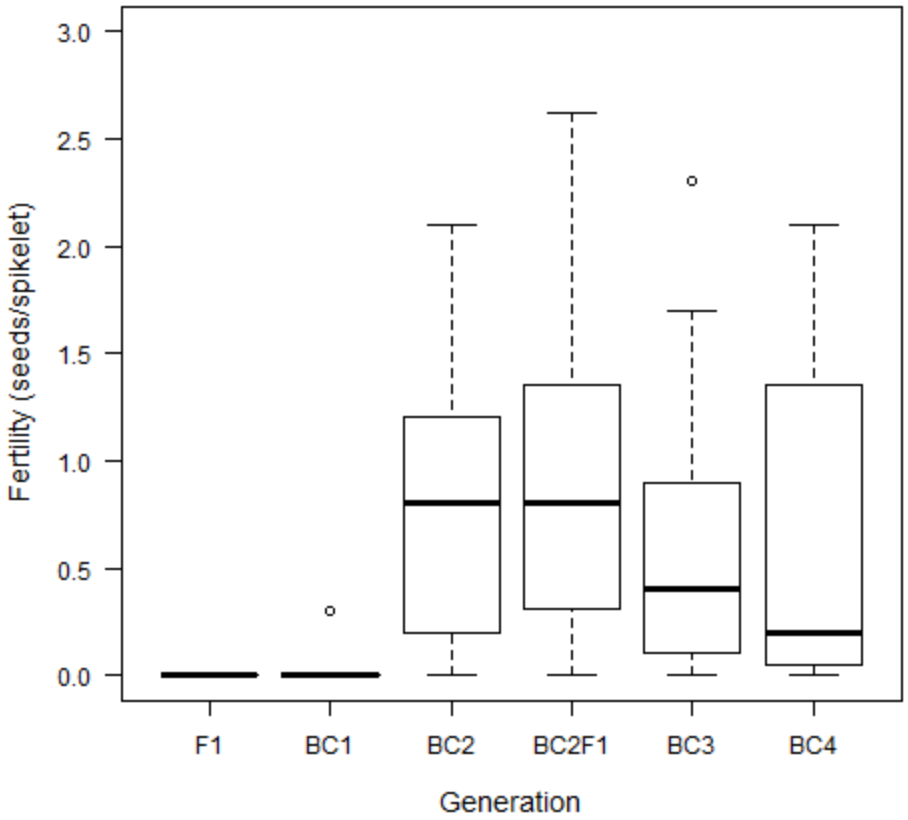


Figure IX-11 Distribution of fertility for each generation

In the box plots, the boundary of the box closest to zero indicates the 25th percentile, a black line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles. Points above the whiskers indicate outliers outside the 90th percentiles.

IX.4. Discussion

The cytoplasm of *T. timopheevii* has been the most widely used in research programs to establish a CMS hybrid wheat system that is commercially acceptable. Unfortunately, no single restorer gene, causing a total and stable restoration, has been discovered, confirming previous studies (Sage, 1972; Wilson, 1968b). However, considering the diversity present in the *Aegilops*-

Triticum complex, one hypothesis was that the cytoplasm of one of these species might allow a total and monogenic restoration. *Ae. speltoides* is considered as the donor of the cytoplasm of *T timopheevii* (Mori et al., 1997) and thus a potential candidate. Like *T. timopheevii*, *Ae. speltoides* carries in its mitochondrial DNA, a sequence equivalent to *orf256*, whose expression results in the male sterile phenotype and expresses a 7 kDa when serving as the cytoplasm donor to wheat (Hedgcoth et al., 2002; Song and Hedgcoth, 1994b). Nevertheless, the presence of a strong gametocidal gene on chromosome 2S of *Ae. speltoides* prevented the transfer of its cytoplasm into wheat (Endo, 1990; Tsujimoto and Tsunewaki, 1984a, 1988) (refer to Chapter I.7). *Ae. speltoides* belongs to the Sitopsis section which also includes *Ae. searsii*, *Ae. bicornis*, *Ae. longissima* and *Ae. sharonensis* (van Slageren, 1994). Male sterile plants with the cytoplasm of *Ae. longissima* and *Ae. sharonensis* have been found but the cytoplasm of *Ae. sharonensis* causes fewer negative effects than the cytoplasm of *Ae. longissima* on the wheat phenotype (Tsunewaki, 2009; Tsunewaki et al., 1976, 1996, 2002). However *Ae. longissima* and *Ae. sharonensis* carry two gametocidal factors, located on group 2 and 4 (Endo, 1982, 1985, 1990; Finch et al., 1984b; Maan, 1975; Miller et al., 1982). A knock-out mutant of the breaker element of the gametocidal chromosome $4S^{sh}$ of *Ae. sharonensis* has been produced and the mutated segment of *Ae. sharonensis* genome has been transferred into chromosome 4B of Chinese Spring (Friebe et al., 2003). This Chinese Spring line with the translocated T4BS.4BL-$4S^{sh}$L chromosome has already been successfully used to remove chromosome $4S^{Sh}$ and to produce wheat/*Ae. sharonensis* translocation lines resistant to leaf, stem or stripe rust (Millet et al., 2014, 2017).

For these reasons, *Ae. sharonensis* was considered as a good candidate to create a new CMS system based on its cytoplasm. A cytoplasmic male sterility system is based on three lines which are the male sterile line, the maintenance line and the restorer line (refer to Chapter I.3.4.4). A crossing programme was carried out in order to transfer the cytoplasm of *Ae. sharonensis* into wheat. Self-fertility of the alloplasmic lines produced has been scored in order to

establish if *Ae. sharonensis* cytoplasm is causing stable and total male sterility. In parallel, production of addition/substitution/translocation lines was carried out. These wheat/*Ae. sharonensis* introgression lines are potential restorer lines against *Ae. sharonensis* cytoplasm.

Production of the F_1 generation

A total of eleven different accessions of *Ae. sharonensis* (ordered from JIC and USDA) were chosen as female parent and pollinated by the T4BS.4BL-4S^{sh}L translocation line, named as CS/*Gc* mutant, produced by Friebe et al., 2003. From 252 crosses a total of 427 F_1 seeds were produced with an average of 1.7 seeds/crossed head. However, seed set varied according to the *Ae. sharonensis* accession. The pollination of fourteen heads of accession 2170007 did not produce any seed while the crosses with accessions 584414, 2170001 and JIC18A500011 were the most successful with 2.90, 2.90 and 3.21 seeds obtained per head crossed respectively. A huge diversity between *Ae. sharonensis* accessions has been reported (Olivera et al., 2010). This might explain the different crossability levels of the various accessions with common wheat. Similar seed set was obtained by pollinating wheat with the pollen of other Aegilops species, e.g. 3.3 seeds/spikelet in the case of *Am. muticum* (King et al., 2017) and 4.9 seeds/spikelet in the case of *Ae. speltoides* (King et al., 2018). Most of the F_1 seeds were shrivelled. Shrivelled seeds might be the result of two possibilities. The first one is the difference of ploidy level between the two parents. The female parent, *Ae. sharonensis*, was diploid ($S^{sh}S^{sh}$) and the male parent, CS/*Gc* mutant, was hexaploid (AABBDD). This difference in ploidy level in the F_1 caused an unbalanced ratio with an excess of the paternal genome which can lead to embryo abortion (Thompson, 1930).This conclusion has been confirmed by the results of Chapters 3 and 5 of this thesis. Nevertheless, in this chapter, the choice of cross direction was imposed by the necessity to work on the cytoplasm of *Ae. sharonensis*, i.e. to use *Ae. sharonensis* as female parent. In some studies, a bridging species is used as the first male parent to reduce the gap between the two parental genomes. For example, *Ae. ventricosa* was firstly crossed with *T. turgidum* (Doussinault et al.,

1983), *Am. muticum* with *T. dicoccum* (Panayotov and Tsujimoto, 1997) and *Ae. umbellulata* with *T. diccocoides* (Sears, 1956b) to produce introgression lines with wheat. However, this strategy involves more back cross generations to eliminate the genome of the bridging parent. The second reason explaining the high proportion of shrivelled F_1 seeds is the presence of the gametocidal gene on chromosome $4S^{sh}$ of *Ae. sharonensis*. It has been shown that *Gc* chromosome $4S^{sh}$ causes chromosome damage in early embryo and endosperm development (King and Laurie, 1993). These post-zygotic barriers led to a very low germination rate of the F_1. Only five percent (twelve seeds) of the F_1 seeds sown, and where the embryo had been rescued on a MS medium, germinated and gave viable F_1 plants. Out of the eleven accessions of *Ae. sharonensis* used as female parents, only the combination with four of them gave viable F_1: 584406, 584408, 2170001 and JIC18A500011.

Production of the back-crossed generation

The F_1 plants were back crossed with the pollen of the CS/*Gc* mutant twice and then Paragon was used as recurrent parent in order to improve the phenotype of the lines with Paragon having been considered as an elite variety in the UK (Angus et al., 2011). Through the back cross generations, an increase in the germination rate (up to 100%) and the success of the crosses (up to 10.48 seeds/spikelet) was observed. The F_1 lines were all self-sterile as were nine of the BC_1 lines produced. Sterility of the early generations is commonly noticed in various wheat/wild relatives back crossing programmes (Grewal et al., 2018a, 2018b; King et al., 2017, 2018). This sterility is the result of the production of gametes with an unbalanced genome (Maan and Sasakuma, 1977). The F_1 plant is composed of a haploid chromosome set of each genome (confirmed by the GISH analysis). Successive back-crosses restored a total set of wheat chromosomes and a segregation of the chromosomes of *Ae. sharonensis* giving rise to gametes with a more normal chromosome behaviour at meiosis. An additional reason for the increase in the success of the crosses could be the absence of the *Gc* chromosome. Only back-crossed lines with the

cytoplasm of accessions 584408 and 2170001 were taken to the BC_5 generation.

Development of male sterile lines

The $4S^{sh}$ chromosome carrying the *Gc* factor, usually transmitted to 98% of the progeny (King et al., 1991), was successfully removed, as also previously reported by Millet et al., 2014 and 2017. While in the previous studies, the lines were based on *T. aestivum* cytoplasm, this work reports the development of alloplasmic wheat BC_4 lines with the cytoplasm of two different accessions of *Ae. sharonensis*, two of which carry the forty-two chromosomes of wheat. The alloplasmic lines were screened for their self-sterility. The two original alloplasmic BC_2 lines, ie. these lines lost the whole *Ae. sharonensis* genome, were self-fertile with 0.27 and 1.68 seeds/spikelet respectively. These results led to the hypothesis that at least one restorer factor against *Ae. sharonensis* cytoplasm was present in the wheat genome, Chinese Spring in this case. The ability of Chinese Spring and other wheat genotypes to restore fertility, has already been reported (Tsunewaki, 2009; Tsunewaki et al., 1976). The wheat recurrent parent was therefore changed to the cultivar Paragon for the production of the BC_3, BC_4 and BC_5 generations (the ability of Paragon to restore *Ae. sharonensis* cytoplasm having not been reported). We noticed a drastic decrease of the male fertility between the BC_2/BC_2F_1 and the BC_3/BC_4 generations. Five BC_3 and fifteen BC_4 alloplasmic lines were male sterile supporting the fact that *Ae. sharonensis* cytoplasm, introduced into common wheat, induced total male sterility (Tsunewaki, 2009; Tsunewaki et al., 1976, 1996). However, these results are only preliminary. More back-crossed generations will have to be produced in order to study the stability of the male fertility, e.g. Tsunewaki (2009) studied the male fertility of alloplasmic lines produced with the different *Aegilops* – *Triticum* complex species after more than 10 generations of back crosses. This provides proof for the stability of the coexistence between the wheat nucleus and the wild species cytoplasm. A back cross programme will also be needed to clean the genetic background of the Chinese Spring genome, which is able to restore fertility. In parallel, other

wheat varieties could be tested as potential non-restorer back grounds. Once a non-restorer wheat back-ground has been established, a proper phenotypic study of the alloplasmic lines will have to be carried out to establish if *Ae. sharonensis* causes deleterious effects. Various morphological and physiological traits will have to be observed as suggested by Tsunewaki et al., (1976) such as heading date, number of tillers, number of spikelets/ear and growth vigour.

Development of wheat/*Ae. sharonensis* introgression lines as potential restorer lines

The second part of a CMS based hybrid system is the development of restorer lines against the cytoplasm causing the male sterility. The natural source of restorer factors is the genome of *Ae. sharonensis* itself. For that reason, a broad set of wheat/*Ae. sharonensis* introgression lines were produced with the genomes of two different *Ae. sharonensis* accessions, 584408 and 2170001. The lines were characterized by GISH analysis. Monosomic addition, substitution and translocation lines were detected. No monosomic wheat/*Ae. sharonensis* addition or substitution lines have previously been produced expect for chromosomes $2S^{sh}$ and $4S^{sh}$ (Zhang et al., 2001). By using the CS/*Gc* mutant line developed by Friebe et al. (2003), the *Gc* chromosome $4S^{sh}$, which is the *Gc* chromosome with the strongest effect, was successfully removed and allowed the isolation of the other chromosomes of *Ae. sharonensis*. A recombination strategy, based on the use of the wheat *ph1b* mutant has previously been used to produce wheat/*Ae. sharonensis* translocations involving chromosomes $1S^{sh}$, $5S^{sh}$ and $6S^{sh}$ (Marais et al., 2010; Millet et al., 2014, 2017). It has been shown that *ph1* mutant lines contain a high level of intergenomic rearrangements, particularly between the A and the D genomes (Sanchez-Moran et al., 2001), which could have a negative impact on the recombination lines produced. Supplementary back-cross generations are thus needed to stabilize the wheat genome. However, cytological studies by C-banding and genotyping studies demonstrated that the genome of *Ae. sharonensis* maintained the chromosome structure of the wheat genome

without any evidence of any chromosomal rearrangements (Friebe and Gill, 1996; Maestra and Naranjo, 1997; Zhang et al., 2001). This result suggests that spontaneous translocations can occur between the wheat and *Ae. sharonensis* genomes. The wheat/*Ae. sharonensis* translocated chromosomes produced here resulted from spontaneous recombination in the presence of the *Ph1* locus, between the B-genome and *Ae. sharonensis*. A more precise characterization of the wheat/*Ae. sharonensis* introgression lines produced could be carried out with the KASP markers developed by the Nottingham/BBSRC Wheat Research Centre (University of Nottingham) and used successfully in Chapter 8. Genotypes BC_2.21 and BC_2.22, which showed the highest restoration of fertility (close to the fertility of the control), carried a translocated wheat/*Ae. sharonensis* chromosome and two complete chromosomes of *Ae. sharonensis,* respectively. It was therefore not possible to conclude which chromosome of *Ae. sharonensis* carries a restorer factor, especially as Chinese Spring carries some restorer factors as well. Moreover, the highest fertility restoration levels in the BC_3 generations were obtained in alloplasmic lines. The highest fertility in the presence of *Ae. sharonensis* (two complete chromosomes) was half of the fertility of the control. In order to test the restoration ability of the wheat/*Ae. sharonensis* introgression lines, several backcrosses would have to be done in order to transfer the *Ae. sharonensis* introgressions into a non-restorer wheat background (wheat background defined during the establishment of the male sterile alloplasmic lines). The fertile lines will carry a restorer factor from *Ae. sharonensis*. In order to optimize the fertility restoration, the segment carrying the restorer factor will need to be transferred into a wheat chromosome and its size will need to be as small as possible to reduce linkage drag.

However, we showed that the transmission rate of the *Ae. sharonensis* genome was very poor. The transmission rate was slightly higher by selfing compared to back crossing. A single complete chromosome of *Ae. sharonensis* was transmitted to the next generation in 44% of the back cross offspring and in 50% of the selfed-progeny (which follow the expected Mendelian values). In

the case of a wheat/*Ae. sharonensis* translocated chromosome the transmission rate was 28 and 39% respectively. The low transmission of the wild genome has been previously reported (Ceoloni et al., 1988; Rubio et al., 1999; Sherman et al., 2001; Whelan, 1988), although the retention rate of the wild genome through the generations was shown to be higher when it was transmitted through the male gametes (Efremova et al., 1996). Stabilization of the genome can be achieved by selfing to reach a homozygous stage when they would be expected to show a higher inheritance. Some homozygous addition and translocation lines were detected in the BC_2F_1 generation. Homozygous translocation lines can be successfully produced using a doubled haploid (DH) technique (Yan et al., 2017). Another possible method to fix the restorer segment of *Ae. sharonensis* would be to produce a translocation line with the long arm of chromosome $4S^{sh}$ carrier of the *Gc* gene. This strategy was successfully developed to stabilize the short and the long arms of chromosome 1U of *Ae. umbellulata* which carries interesting genes for baking quality (King et al. 1992). However, it is essential to evaluate the consequence of such chromosomes on the phenotype of the line.

Chinese Spring, another source of restorer factors against *Ae. sharonensis* cytoplasm

Chinese Spring has been shown to be able to restore *Ae. sharonensis* cytoplasm (Tsunewaki, 2009; Tsunewaki et al., 1976). In this study, we found fertile alloplasmic lines without any *Ae. sharonensis* chromosomes. The highest fertilities found were 1.7 seeds/spikelet for the BC_2 generation, 2.62 seeds/spikelet for the BC_2F_1 generation, 2.32 seeds/spikelet for the BC_3 generation and 2.13 seeds/spikelet for the BC_4 generation. Selfed seeds were present up to the top of the spike, often a sign of a high level of fertility restoration. A decrease in the level of fertility was noticed between the BC_2/BC_2F_1 and the BC_3/BC_4 generations which differ by the wheat recurrent parent from Chinese Spring to Paragon. This supports the hypothesis that Chinese Spring might carry strong restorer factors which are not present in the Paragon genome. However, limited information is known about the number of

restorer factors and their localisation on the genome. A mapping population could be developed by crossing a male sterile alloplasmic line with a male fertile alloplasmic line in order to carry out QTL analysis to localize the restorer factor(s) on the Chinese Spring genome. Another solution is the pollination of the sterile alloplasmic line by Chinese Spring chromosome arm deletion lines. However, we could suppose that one restorer factor is present on chromosome 1B. It has been shown that the short arm of chromosome 1B of Chinese Spring carries restorer genes against the cytoplasm of different wild species. These genes have been named as *Rf3* for *T. timopheevii* (Kučera, 1982), *Rfv1* for *Ae. kotschyi* (Mukai and Tsunewaki, 1979), *Rfm1* for *Am. muticum* (Tsujimoto and Tsunewaki, 1984b) and *Rfn1* for *Ae. uniaristata* (Mukai, 1983). In addition, it has been shown that the genes *Rfv1*, *Rfm1* and *Rfn1* were located at the same locus, named as Rf^{multi} (Tsunewaki, 2015).

Conclusion and perspectives

The difficulties encountered in producing a monogenic restorer causing stable and total restoration of *T. timopheevii* cytoplasm encouraged work to discover new sources of male sterile cytoplasm. One potentially interesting source is the cytoplasm of *Ae. sharonensis*. However, the presence of a gametocidal gene on chromosome $4S^{sh}$ has prevented the production of alloplasmic lines with the wheat nuclear genome and the cytoplasm of *Ae. sharonensis*. By using the T4BS.4BL-$4S^{sh}$L translocation line (CS/*Gc* mutant), with the mutated allele of the *Gc* gene transferred to a Chinese Spring background (Friebe et al., 2003), the *Gc* chromosome was successfully removed. Alloplasmic lines with the cytoplasm of two different accessions of *Ae. sharonensis* were developed. Male sterility was expressed in some alloplasmic lines, depending on the wheat back-ground. Our preliminary results showed that Chinese Spring carried some strong restorer factor(s). Further investigations are needed, however, to determine the number, the localization and the effect of the restorer genes. In parallel to the development of alloplasmic lines, a set of introgression lines including addition, substitution and translocation lines have been developed.

These lines are potential restorers for the cytoplasm of *Ae. sharonensis* and also for other male sterile cytoplasm such as *T. timopheevii*.

X. Chapter 10: General Discussion

The commercial development of hybrid wheat is mainly dependant on the establishment of an efficient seed production system (Kempe and Gils, 2011). Many different methods have been developed to produce hybrid wheat but the most current system used worldwide is based on male sterile cytoplasm (Whitford et al., 2013). This system relies on two different components, i.e. the cytoplasm causing male sterility in the female line and the restorer factor carried by the male line insuring the fertility of the hybrid F_1. Both male sterility and fertility restoration have to be total and stable to not counteract the effects of heterosis on grain yield, which is a major motivation for hybrid wheat breeding. The wild relative diversity is a useful reservoir for the two components of the CMS system, i.e. the cytoplasm and the restorer factor (Hajjar and Hodgkin, 2007). Commercial varieties of several crops have been developed based on the cytoplasm of a wild relatives, such the wild species *Helianthus petiolaris* for sunflowers (Le Clercq, 1969), wild rice *Oryza rufipogon* cytoplasm for cultivated rice (Luo et al., 2013) and Pampa, an Argentinian landrace, for rye (Geiger and Schnell, 1970). Restorer genes were also transferred from the wild genome to the cultivated genome in the first two cases (Hu et al., 2016; Kusterer et al., 2005) whereas restorer genes were introduced from Iranian rye accessions for the rye system (Stracke et al., 2003).

CMS in wheat is mainly based on the cytoplasm of *T. timopheevii*, a tetraploid wheat. This cytoplasm has been selected as it does not cause important deleterious effects on the phenotype once transferred into common wheat (Tsunewaki et al., 1976; Wilson and Driscoll, 1983). Hence the decision to work, in the first instance, with the cytoplasm of *T. timopheevii*. The remaining barrier to the commercial exploitation of a hybrid wheat CMS based on the cytoplasm of *T. timopheevii* is the development of a monogenic restorer line causing total fertility (Whitford et al., 2013). Based on the knowledge of a high conservation of genome collinearity within the tribe Triticeae (Devos and Gale,

2000), led to the hypothesis that all the wheat wild relatives are potentially sources of restorer genes against *T. timopheevii*. Thus, the main objective of the thesis was to study the ability of the wheat wild relatives to restore *T. timopheevii* cytoplasm. We identified new restorer factors on chromosomes 4R of *S. anatolicum*, $1A^t$ and 6G of *T. timopheevii*, $5S^{sh}$ and $6S^{sh}$ of *Ae. sharonensis*.

Identification of potential donors is the preliminary step in the transfer of an interesting trait (Sharma et al., 2013). In order to evaluate the whole genome in a quick and easy way, wheat/wild relative amphidiploids were used as parental lines to overcome the usual reproductive barriers encountered when directly crossing the wild relative and the cultivated species (Chang and Jong, 2005). Nevertheless, the multiple post-zygotic barriers encountered during the production of the F_1, i.e. the high percentage of shrivelled seeds and the low germination rate, made the screening of the wild genomes extremely laborious and allowed the screening of only five wild species out of the thirteen species initially selected. In addition, the presence of a complete set of wheat chromosomes and a haploid set of wild relative chromosomes in the F_1, caused high genomic instability and led to hybrid sterility masking the potential presence of restorer factors. This reflects the problem encountered during the development of Triticale. Chromosome doubling was a mandatory step to produce fully fertile plants (Lorenz and Pomeranz, 1974). Despite these barriers, the ability of *S. anatolicum* to partially restore the cytoplasm of *T. timopheevii* was established for the first time.

The first hybridization product between wheat and the amphidiploid cannot be used directly. However, the F_1 can be considered as a starting point for the development of introgression lines, e.g. this strategy was used for the development of wheat/*Th. bessarabicum* introgression lines (Grewal et al., 2018a). In analogy, wheat/S*ecale* and wheat/*Ae. sharonensis* amphidiploids were used to produce introgression lines.

Compared to the amphidiploid which carries the complete parental genomes, monosomic wheat/wild relative additions or substitutions carry a single complete chromosome of the wild species. This material is particularly interesting for two reasons. The first is the reduction of both the reproductive barriers and linkage drag by the reduction in the amount of the wild genome present in the line. In the different crossing programs in this thesis, we showed an increase in the germination rate and the success of the crosses through the generations. For example, chromosome 2R of rye is known to reduce the fertility of a wheat line into which it has been introgressed (Schlegel, 2013). Secondly, this type of material is very useful to individually study each wild chromosome for their potentially interesting agronomic traits (Gong et al., 2017; Lammer et al., 2004; Wang et al., 2010, 2011) and for fertility restoration ability in particular (Castillo et al., 2014; Curtis and Lukaszewski, 1993). The isolation of the 4R restorer chromosome of *S. anatolicum* increases the fertility level from 0.33 to 1.75 seeds/spikelet (in comparison with the fertility level obtained in the F_1 hybrid resulting from the cross between wheat and the wheat/*S. anatolicum* amphidiploid). For the first time, the wheat/*Ae. sharonensis* introgression lines produced allowed the detection of the presence of restorer factors on chromosomes $5S^{sh}$ and $6S^{sh}$ of *Ae. sharonensis*. The crosses using addition or substitution lines were more successful but the segregation of the wild chromosome and its elimination from lines again prevent the formation of stable introgression lines (Chang and Jong, 2005). We showed that the restorer chromosome was maintained in 30% of the offspring of *S. anatolicum* and 40% of the offspring of *Ae. sharonensis*.

The most advanced material is the wheat/wild relative translocation lines, where a segment of the wild relative genome has been incorporated into the wheat genome via meiotic recombination. Translocation lines have been recognized as a means for providing the most promising pathway for the utilization of wild germplasm by providing a stable resource with reduced linkage drag (Qi et al., 2007). The centric breakage-fusion behaviour of univalents can be exploited to produce spontaneous translocation lines (Sears,

1952). In addition or substitution lines, unpaired chromosomes are present as univalents, and thus have a chance to mis-divide and then re-fuse, giving rise to Robertsonian whole arm translocations. This phenomenon allows the production of translocated chromosomes with the genome belonging to the tertiary gene pool of wheat and less disposed to recombine with the wheat genome because of the poor homology. Centric-breakage fusions have been reported in wheat/*Th. ponticum* (Jan et al., 1981; Li et al., 2017b) and wheat/rye (Carvalho et al., 2009; Mettin et al., 1973; Wang et al., 1995; Zeller and Koller, 1981). and in this work the production of wheat/*S. anatolicum* translocated chromosomes.

Translocations in other positions, i.e. non centric, are not common since the homoeologous chromosomes between wheat and wild species in wide hybrids show a low pairing level due to the action of the *Ph1* locus on chromosome 5B of wheat, which restricts the production of wheat/wild relatives translocations (Martinez-Perez et al., 2008; Sears, 1976). Thus a crossing program was carried out to produce wheat/*Secale* translocation lines using the *ph1* mutant line, with a particular interest in chromosomes 4R and 6R which are known to carry restorer factors. However, no translocation line was detected, probably because of the very low homology between wheat and *Secale* genomes. Lukaszewski (2000) reported the successful use of the *ph1* mutant line to reduce the size of a segment of 1R of rye. However, chromosome 1R of rye shows complete homology to the wheat chromosomes of linkage group 1, which is not the case with 4R and 6R (Devos et al., 1993).

A crossing program based on the *ph1* mutant line could be carried out to introgress a smaller fragment carrying the restorer factors from chromosomes $5S^{sh}$ and $6S^{sh}$ of *Ae. sharonensis* in wheat. Such an introgression program seems likely to be more successful than the one involving the *Secale* genomes because of the higher similarity of the genomes of *Ae. sharonensis* and wheat. The mutant with the *ph1* locus deleted was successfully used to produce recombinant lines with small genome segments of *Ae. geniculata* (Kuraparthy

et al., 2007) and *Th. bessarabicum* (Grewal et al., 2018a), species showing a high collinearity with wheat. The presence of a spontaneous wheat/*Ae. sharonensis* translocation chromosome was detected, despite the presence of the *Ph1* locus. However, the size of the fragment introgressed was still large. A cross done with the *ph1* mutant line could reduce the size of the segment as reported by Millet et al., (2017). The expected consequence of the production of translocation lines with a segment carrying the restorer factor is the increase in the genomic stability of the lines and thus a higher and more stable restoration of fertility.

Such work was done to produce wheat/*T. timopheevii* translocation lines by the WRC. These lines were evaluated for their restoration ability. Chromosomes $1A^t$ and 6G were shown to be able to partially restore the cytoplasm of *T. timopheevii*. Out of all the material used and the results presented in this thesis, this was the most developed material which could be used in a pre-breeding program by the seed company. Only a few more generations of back-crosses are needed to reduce the size of the fragment carrying the restorer factor and to convert the lines into an elite background.
One essential criteria for the efficient use of an introgressed wild genome segment is the availability of tools to track this segment though the generations. This is a basic requirement for the development of a pre-breeding population (Dempewolf et al., 2017). The cytogenetic methods developed are a very good tool for a first academic approach and it is useful to have a visual representation of the genomic content of the lines. GISH allows the differentiation of the different genomes present in a line, from a small segment to a complete chromosome. However, if two genomes are to similar, e.g. the A-genome of wheat and the A^t genome of *T. timopheevii*, GISH will not allow their distinction. FISH techniques give supplementary information on the identity of the linkage group involved if the segment present carries a specific pattern. However, the first requirement is the development of a karyotype of the wild species using probes that are chromosome specific but not all the wild species have been karyotyped as yet, e.g. *Ae. sharonensis*.

In this thesis we showed some of the limits of GISH and FISH. These methods are highly time and labour consuming, preventing the screening of a large population, and thus decreasing the probability of detecting a single event, i.e. a recombination between the wheat and wild relative genomes. The crossing programme to develop wheat/*Secale* introgression lines using the *ph1* mutant is one good example. The limited number of plants (less than one hundred) screened could be one explanation for why no wheat/*Secale* translocated chromosomes were detected. Genotyping tools allow the wheat/wild relatives introgression lines produced to be characterized in a higher through put way. In this thesis, we used the KASP markers developed at Nottingham during summer 2018 and designed to be polymorphic between wheat and ten wild relative genomes. The results were consistent with the cytological analysis for both the *Secale* and *Ae. sharonensis* introgression lines. These markers allowed us to characterize a larger number of lines. However, we showed the need for the cytological results to determine the nature of the introgression, i.e. addition, substitution or translocation when the introgression was heterozygous although the markers are fully informative when the wild genome introgression is homozygous and has replaced its homoeologous wheat linkage group. Additional genotyping work is needed, the ultimate goal being the development of allele specific markers to precisely characterize precisely the lines produced and to facilitate a breeding program.

The last part of the project focused on *Ae. sharonensis* as a potential source of sterile cytoplasm. The work presents only preliminary results and additional back cross generations are needed to produce interesting material. However, the exploration of new sources of male sterile cytoplasm is essential. Firstly, the cytoplasm of *Ae. sharonensis* might be easier to restore than that of T. *timopheevii*. Secondly, the use of a single source of sterile cytoplasm increases genetic vulnerability. The most famous example of this was the outbreak of Southern corn blight due to *Helmintosporium maydis* on US maize hybrids carrying T-type cytoplasm (Hooker, 1974).

The initial question remains unsolved: how many restorer genes are needed to restorer the fertility? The initial results all indicate the same as previous work, at least two genes are needed (Geyer et al., 2018; Sage, 1972; Wilson, 1968a; Würschum et al., 2017b). The same observation has been made in hybrid Triticale based on the cytoplasm of *T. timopheevii* for which at least two restorer factors are needed (Góral et al., 2010), located on the rye and wheat genomes (Stojalowski et al., 2013). These results increase the difficulty of the development of restorer lines combining two or three restorer genes (Chen, 2003). Additional generations of crossing are needed to pyramid the genes in the same lines and development of allele specific markers are essential (Kumar Malav et al., 2016). The difficulty might also be increased by the fact that the effect of the restorer genes might not be additive. Geyer et al. (2018) highlighted this phenomenon in the case of the combination of the restorer genes *Rf1* and *Rf4* from wheat. However, it might be possible that the reduction of the wild chromatin, in particular *Ae. sharonensis* or *T. timopheevii*, in the restorer line after successive back-crosses could lead to a total restoration in the presence of a single dominant restorer gene by removing all the deleterious genes. Further studies are needed on the material produce to explore this hypothesis.

The work done through this PhD has highlighted the difficulty involved in introgressing wild genetic diversity into wheat without different negative aspects, e.g. postzygotic barriers or linkage drag. The production of a successful and stable system will not be effected immediately. More than 3,200 crossings were carried out and more than 7,800 seeds were produced during the four years which has produced some preliminary materials concerning the genomes of *Ae. sharonensis* and *T. timopheevii*. However, placing this work in the breeding context, several decades are usually needed to establish an efficient system.

One successful example of the use of genetic diversity for the development of a hybrid system based on male sterile cytoplasm and developed in spite of

reproductive barriers (and encompassing many of the requirements discussed above), is the INRA Ogura based hybrid rapeseed (*Brassica napus*) which took over thirty years to develop before delivering a commercial variety. Two thirds of the varieties recommended for the UK for the year 2017-2018 were based on this CMS system (cereals.ahdb.org.uk/varieties). The cytoplasm of the radish *Ogura* (Ogura, 1968) was transferred to *B. napus* causing complete sterility (Bannerot et al., 1974). However, the alloplasmic lines showed chlorophyll deficiency (Pelletier et al., 1983) which was overcome by carrying out protoplast fusion between male sterile *B. napus* and normal *B. napus* (Jarl and Bornman, 1988; Pelletier et al., 1983). The mitochondrial genes causing the male sterility was identified and named as *orf138* (Bonhomme et al., 1991, 1992). A nuclear restorer gene, *Rfo*, has also been transferred from the radish to *B. napus* through interspecific hybridization (Heyn, 1976; Pelletier et al., 1983). The introgressed segment of radish also contained an unwanted gene responsible for seed glucosinolate synthesis. After successive back crosses, the size of the introgressed segment was reduced and the deleterious effects removed (Delourme et al., 1991, 1998; Giancola et al., 2003). The *Rfo* gene has been cloned successfully (Brown et al., 2003; Desloire et al., 2003) and allele-specific markers have been developed to track the restorer allele through the hybrid breeding program (Hu et al., 2008).

We have shown in this thesis different methods to exploit the genetic interspecific diversity of wheat through hybridization. However, in addition to the barriers already discussed above, non-random recombination of the wheat and wild relative genomes can also make it impossible to introgress the restorer gene into wheat (Lukaszewski et al., 2004). An alternative method to make use of the genetic diversity is the genome editing technique CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats). CRISPR-Cas9 is based on the formation of a complex between the Cas9 protein, which has the capacity to cleave DNA, and a small RNA which is complementary to the target sequence of the genome and it has been successfully set up in wheat (Gil-Humanes et al., 2017; Kim et al., 2018). Targeted mutation and base editing

(replacement of a specific base) can be achieved. Successful gene inactivation has been achieved in wheat leading to the development of a genic male sterile (Singh et al., 2018), powdery mildew resistant (Wang et al., 2014b) and a low gluten wheat (Sánchez-León et al., 2018). CRISPR-Cas9 can thus target alleles which are absent in wheat but present in a relative species (Andersen et al., 2015), i.e. the knowledge of the restorer alleles of chromosomes $1A^t$ and 6G of *T. timopheevii*, $5S^{sh}$ and $6S^{sh}$ or *Ae. sharonensis* and 4R and 6R of rye is a prerequisite for the use of the technique. An extreme example is the de novo domestication of wild species by editing the main domestication genes and introducing new allele diversity for biotic and abiotic resistance as has been studied in tomato (Zsögön et al., 2017). However, the development of such material is dependent on the current laws and regulations, for example the development and use of gene-edited plants is prohibited within the European Union (Jouanin et al., 2018).

Appendix

Appendix 1 Buffer and Solution protocols

1 x T.E.

For 100 mL solution:

- De-ionised water: 90 mL
- 10 x T.E.: 10 mL

10 x T.E.

To make a solution with a pH of 7.5:

- 100 mM of Tris
- 10 mM of EDTA

2x SSC

For one litre of 2 x SSC:

- Measure out 900 mL of de-ionised water
- Add 100 mL of 20 x SSC

20 x SSC

For one litre of 20 x SSC:

- Weigh out 175.3 g of NaCl and 88.2 g of $Na_3C_6H_5O_7$ (Trisodium citrate)
- Dissolve in 800 mL of de-ionised water
- Adjust the pH to 7.0 using drops of hydrochloric acid
- Adjust the volume to 1 L by adding additional de-ionised water
- Autoclave to sterilise

3M NaOAC (pH 5.2) (sodium acetate)

For 100 mL of NaOAc (pH5.2):

- Dissolve 40.8 g of powdered NaOAc in 70 mL of de-ionised water
- Adjust the pH to 5.2 by adding glacial acetic acid

- Add water to bring the total volume of the solution to 100 mL
- Check pH once more with a pH meter
- Store at room temperature indefinitely

6M ammonium acetate

For 50 mL solution:

- Dissolve 23.124 g of 6M $NH_4C_2H_3O_2$ in 50 mL of de-ionised water
- Store at 4°C

ClNaO (sodium hypochlorite)

For 50 mL 5% solution:

- Stock solution (10%): 25 mL stock
- Water: 25 mL
- Tween 20: 1-2 drops

DAPI

For a solution of 20 mL, 1:3 ratio

- DAPI: 5 mL
- 1 x TE: 15 mL

Digestion enzyme (1% pectolyase Y23 and 2% cellulose Onozuka R-10)

For 10 g of the enzyme solution:

- Pectolyase Y-23 (1% w/w) : 0.1g
- Cellulase Onozuka R-10 (2% w/w): 0.2g
- 1 X Citric Buffer (pH5.5) (Appendix 1): 9.7g

Solution divided into 15-20 µL aliquots in 0.5 mL tube and stored at -20°C

DNA extraction buffer

For 100 mL solution:

- Tris-HCL (pH 7.5): 10 mL (1.0 M)

- EDTA (pH 8.0): 10 mL (0.5 M)
- 10% SDS : 12.5 mL
- De-ionised H2O : 67.5 mL

MS medium

For 1L solution

- Murashige and Skoog dried medium: 4.41 g
- Sucrose (3%): 30 g
- Agar (8%) 8 g
- Water: up to 1 L

ROX PACE mix:

- Aliquot Rox-free PACE mix (Applied Biosystems) from a bottle of 250 mL to 25 mL portions in 50 mL tubes
- Make 500 µL of 1:10 dilution of the Rox stock (Applied Biosystems) by adding 50 µL of Rox Stock to 450 µL dH_2O, mix well
- Add 75 µL of 1:10 dilution of Rox to a 25 mL aliquot of Rox-free PACE, mix thoroughly and label the tube clearly Rox added
- Store at -20°C

SS DNA (single stranded DNA) (140 µL/mL)

For 50 mL solution:

- 50x TAE (pH 6.3): 5 mL
- De-ionised water: 44.3 mL
- 10 mg/ml SS DNA: 0.7 mL
- Store at 4^{o}C

Appendix 2 Pedigree of the wheat/rye introgression lines developed by the WRC used a pollen donor

CS abbreviation for Chinese Spring, Pg abbreviation for Paragon, genotype underlined produce fertile F_1

Code	Pedigree
BC2-306C	(((CS *ph1* mutant x *S. cereale* 428372) x Pg) x Pg)
BC2-306D	(((CS *ph1* mutant x *S. cereale* 428373) x Pg) x Pg)
BC2-306E	(((CS *ph1* mutant x *S. cereale* 428373) x Pg) x Pg)
BC2-730A	(((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg)
BC2-731A	(((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg)
BC2-733A	(((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg)
BC2-734C	(((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg)
BC2-736A	(((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg)
BC2-736B	(((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg)
BC2-736C	(((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg)
BC2-738B	(((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg)
BC2-738D	(((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg)
BC2-739B	(((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg)
BC2F1-254B	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) self)
BC2F1-254C	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) self)
<u>BC2F1-254D</u>	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) self)
BC2F1-254E	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) self)
BC2F1-254F	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) self)
BC2F1-254G	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) self)
BC2F1-68A	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) self)
BC2F1-68B	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) self)
BC3-351E	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x CS) x Pg)
BC3-351F	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x CS) x Pg)
BC3-351G	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x CS) x Pg)

BC3-351H	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x CS) x Pg)
BC3-636D	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-637A	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-638A	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-638B	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-638D	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-638E	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-640A	((((CS *ph1* mutant x *S. cereale* 428373) x Pg) x Pg) x Pg)
BC3-640B	((((CS *ph1* mutant x *S. cereale* 428373) x Pg) x Pg) x Pg)
BC3-640C	((((CS *ph1* mutant x *S. cereale* 428373) x Pg) x Pg) x Pg)
BC3-640D	((((CS *ph1* mutant x *S. cereale* 428373) x Pg) x Pg) x Pg)
BC3-640E	((((CS *ph1* mutant x *S. cereale* 428373) x Pg) x Pg) x Pg)
BC3-640F	((((CS *ph1* mutant x *S. cereale* 428373) x Pg) x Pg) x Pg)
BC3-640G	((((CS *ph1* mutant x *S. cereale* 428373) x Pg) x Pg) x Pg)
BC3-643A	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) x Pg)
BC3-644A	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-644B	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-644C	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-645A	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-645B	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-645C	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-648B	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-648C	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-648D	((((CS *ph1* mutant x *S. cereale* 390383) x CS) x Pg) x Pg)
BC3-649A	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) x Pg)
BC3-649B	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) x Pg)
BC3-649C	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) x Pg)
BC3-658B	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-659D	((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg)
BC3-662A	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) x Pg)
BC3-662B	((((CS *ph1* mutant x *S. cereale* 390382) x Pg) x Pg) x Pg)

BC3F1-180B	(((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg) self)
BC3F1-182B	(((((CS *ph1* mutant x *S. cereale* 390382) x CS) x Pg) x Pg) self)

Appendix 3 Pedigree of the wheat/*T. timopheevii* introgression lines developed by the WRC used a pollen donor

CS abbreviation for Chinese Spring, Pg abbreviation for Paragon, Pv for Pavon 76, Hg for Highbury

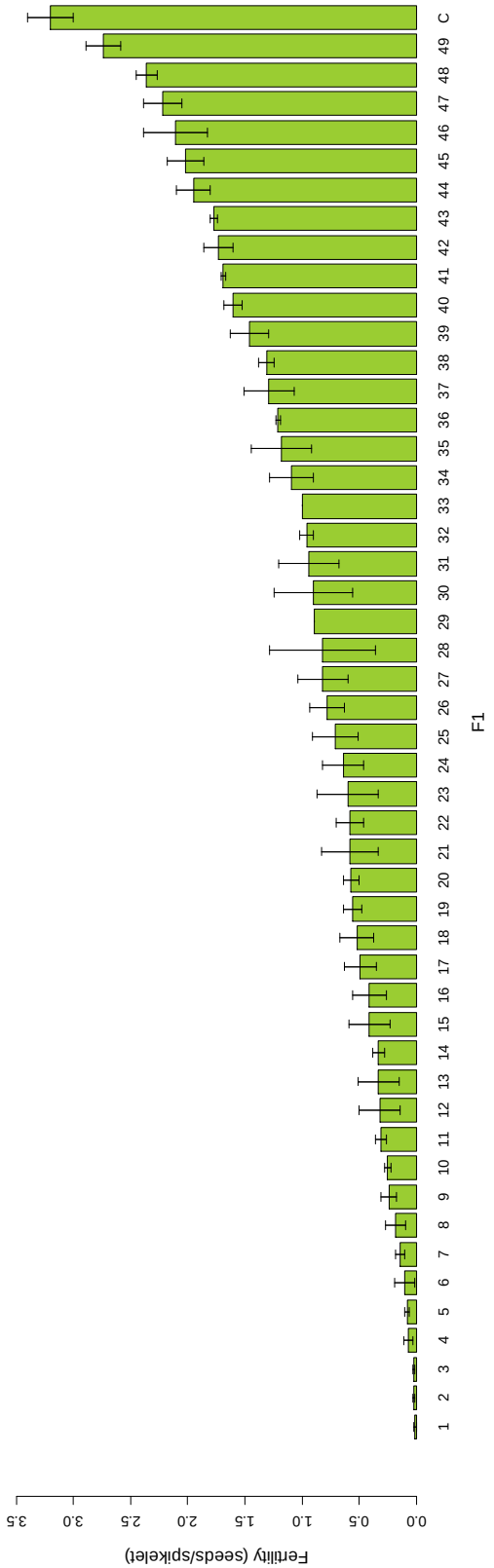
Code	Pedigree
BC2F2-657-4	(((((CS *ph1* mutant x *T. timopheevii*) x Hg) x Pg) self) self)
BC2F2-658-2	(((((CS *ph1* mutant x *T. timopheevii*) x Hg) x Pg) self) self)
BC2F2-659-8	(((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) self) self)
BC2F2-660-2	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self)
BC2F2-660-3	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self)
BC2F2-660-4	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self)
BC2F3-2-2	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self) self)
BC2F3-6-3	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self) self)
BC2F3-6-6	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self) self)
BC2F3-6-7	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self) self)
BC2F3-7-1	((((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self) self)
BC2F3-7-4	((((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self) self)
BC2F3-7-5	((((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self) self)
BC2F3-7-6	((((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self) self)
BC2F3-7-7	((((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self) self)
BC2F3-7-8	((((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) self) self) self)
BC3F1-188-2	(((((CS *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)
BC3F1-192-2	(((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)
BC3F1-193-1	(((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)
BC3F1-193-4	(((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)
BC3F1-193-5	(((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)
BC3F1-193-6	(((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)
BC3F1-194-1	(((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)
BC3F1-194-2	(((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)
BC3F1-194-4	(((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)
BC3F1-194-6	(((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)

BC3F1-194-7	(((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)
BC3F1-197-1	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-198-1	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-199-1	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-199-2	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-199-3	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-199-4	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-199-5	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-200-1	(((((CS *ph1* mutant x *T. timopheevii*) x Pv) x Pg) x Pg) self)
BC3F1-200-3	(((((CS *ph1* mutant x *T. timopheevii*) x Pv) x Pg) x Pg) self)
BC3F1-200-4	(((((CS *ph1* mutant x *T. timopheevii*) x Pv) x Pg) x Pg) self)
BC3F1-200-5	(((((CS *ph1* mutant x *T. timopheevii*) x Pv) x Pg) x Pg) self)
BC3F1-200-6	(((((CS *ph1* mutant x *T. timopheevii*) x Pv) x Pg) x Pg) self)
BC3F1-200-7	(((((CS *ph1* mutant x *T. timopheevii*) x Pv) x Pg) x Pg) self)
BC3F1-200-8	(((((CS *ph1* mutant x *T. timopheevii*) x Pv) x Pg) x Pg) self)
BC3F1-201-1	(((((CS *ph1* mutant x *T. timopheevii*) x Pv) x Pg) x Pg) self)
BC3F1-201-3	(((((CS *ph1* mutant x *T. timopheevii*) x Pv) x Pg) x Pg) self)
BC3F1-201-5	(((((CS *ph1* mutant x *T. timopheevii*) x Pv) x Pg) x Pg) self)
BC3F1-202-1	(((((CS *ph1* mutant x *T. timopheevii*) x Pv) x Pg) x Pg) self)
BC3F1-202-5	(((((CS *ph1* mutant x *T. timopheevii*) x Pv) x Pg) x Pg) self)
BC3F1-203-1	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-203-2	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-203-3	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-203-4	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-203-5	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-203-6	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-203-7	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-203-8	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-204-1	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-204-2	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-204-3	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-204-5	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-204-6	(((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)

BC3F1-207-1	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-207-7	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F1-208-1	(((((CS *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)
BC3F1-208-6	(((((CS *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) self)
BC3F1-210-3	(((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self)
BC3F2-57-1	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-57-2	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-57-3	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-57-4	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-57-5	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-57-6	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-57-7	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-57-8	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-58-1	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-58-2	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-58-3	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-58-5	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-58-6	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-58-7	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-58-9	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-59-4	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-61-2	((((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-61-3	((((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-61-4	((((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-61-5	((((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-61-6	((((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-62-5	((((((CS *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-70-6	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-71-1	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-71-2	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-71-3	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-71-4	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-71-6	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)

BC3F2-71-7	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-72-1	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-72-2	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-72-3	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-72-6	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC3F2-72-7	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) self) self)
BC4F1-74-1	((((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) x Pg) self)
BC4F1-74-2	((((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) x Pg) self)
BC4F1-74-3	((((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) x Pg) self)
BC4F1-74-5	((((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) x Pg) self)
BC4F1-74-6	((((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) x Pg) self)
BC4F1-74-7	((((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) x Pg) self)
BC4F1-75-2	((((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) x Pg) self)
BC4F1-75-4	((((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) x Pg) self)
BC4F1-75-5	((((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) x Pg) self)
BC4F1-77-2	((((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) x Pg) self)
BC4F1-77-5	((((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) x Pg) self)
BC4F1-77-7	((((((Pg *ph1* mutant x *T. timopheevii*) x Hg) x Pg) x Pg) x Pg) self)
BC4F1-78-4	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) x Pg) self)
BC4F1-79-1	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) x Pg) self)
BC4F1-79-3	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) x Pg) self)
BC4F1-80-2	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) x Pg) self)
BC4F1-80-3	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) x Pg) self)
BC4F1-80-4	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) x Pg) self)
BC4F1-80-5	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) x Pg) self)
BC4F1-80-6	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) x Pg) self)
BC4F1-80-7	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) x Pg) self)
BC4F1-80-8	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) x Pg) self)
BC4F1-81-1	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) x Pg) self)
BC4F1-81-8	((((((Pg *ph1* mutant x *T. timopheevii*) x Pg) x Pg) x Pg) x Pg) self)

Appendix 4 Number of seeds/spikelet set by the fertile F_1 plants (CMS x *T. timopheevii* introgression lines)



Genotype number 1 to 49 are the F_1 lines, the control is designated by C

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