The development of wheat/*Thinopyrum elongatum* lines produced via a whole genome shotgun approach and characterisation via cytogenetics and molecular marker analysis

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BSc (Hons)

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

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September 2018

Abstract

Providing a sustainable supply of wheat for a growing global population, whilst managing additional challenges such as climate change and land availability, is a huge challenge for breeders. The two hybridisation events that occurred during wheat's evolution and the subsequent genetic bottleneck, combined with over a century of intensive selection has severely limited the genetic diversity available in the wheat gene pool, directly limiting any improvements breeders can make to increase yield to meet future demand.

Thinopyrum elongatum 401007 is a wild relative of wheat known to contain a plethora of genes beneficial for abiotic and biotic stress tolerance. Genes for leaf rust resistance (*Lr19, Lr24, Lr29*), stem rust resistance (*Sr24, Sr25, Sr43*) and powdery mildew resistance (*Pm51*) have already been exploited. Therefore, *Th. elongatum* 401007 (2n = 10x = 70 (+2)) is being used in this programme to develop chromosome introgression lines in bread wheat, with the aim to increase the genetic diversity available for future breeders. The action of wheat *ph1/ph1* deletion mutants is exploited to enable initial wide interspecies crosses.

The Nottingham BBSRC WRC shotgun breeding programme has successfully generated over 8,000 crossed seed and over 55,000 self-fertilised seed from the wheat/*Th. elongatum* breeding pipeline and has reached the BC_5 and BC_5F_1 generation.

From these lines, GISH has identified 143 introgressions from 327 back-crossed lines. Multi – colour GISH has also shown a closer relationship between the B and A genomes of wheat with the E genome of *Th. elongatum* than previously known. Mc-GISH was also used to investigate the genomic constitution of *Th. elongatum* 401007.

In total, 263 Affymetrix SNP markers have been validated and used to identify 202 introgressions from 352 back-crossed lines. They have also been used to produce a putative linkage map of the E genome. A previously unknown inversion of chromosome 7E in comparison to 7D was discovered and the 4/5 translocation known to be present in wheat was shown to be absent in the genome of *Th. elongatum*. Development and initial testing of KASP markers is shown and ongoing.

Acknowledgements

I would like to start by thanking my partner, Ben Daly, for his continuous support and encouragement throughout this PhD; I would not have completed this without him. I would also like to thank my family and friends, most notably: Alistair, Sophie, Jake, George, Lydia, Orla, Caz, Sophie and Livvy; for keeping me smiling through the ups and downs of a PhD and ensuring I maintained a healthy work-life balance through-out the last four years.

Very special thanks for all members, past and present, of the King Research Group for their friendship and support. In particular, thanks to my supervisors Dr Julie King and Prof Ian King, for their continuous support and guidance on all aspects of this PhD research. Special thanks to my fellow PhD researcher Jack Heath for his friendship throughout the last four years. My heart-felt gratitude must be given to Dr Surbhi Grewal for her genotyping expertise, to Dr Stella Edwards for her technical support and laboratory expertise, to Dr Caiyun Yang for her cytogenetic expertise and to Duncan Scholefield and Stephen Ashling for their invaluable technical support.

A final thanks to those who have helped me throughout this period at any time and whom I have forgotten to mention.

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List of Abbreviations

Abbreviation	Description
Acc.	Accession
AFLP	Amplified Fragment Length Polymorphism
BBH	Best BLAST hit
BBSRC	Biotechnology and Biological Sciences Research Council
BYDV	Barley Yellow Dwarf Virus
CRBT	Call rate below threshold
Cs94	Chinese Spring 94
Cv	Cultivar
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribo-nucleotide triphosphates
FAO	Food and Agriculture Organisation of the United Nations
FISH	Fluorescent *in situ* hybridisation
FHB	Fusarium Head Blight
GBS	Genotyping by sequencing
Gc	Gametocidal chromosome
GISH	Genomic *in situ* hybridisation
IWGSC	International Wheat Genome Sequence Consortium
JIC	John Innes Centre
KASP	Kompetitive allele-specific PCR
LOD	Log odds
MAS	Marker assisted selection
mcFISH	Multi-colour fluorescent *in situ* hybridisation
mcGISH	Multi-colour Genomic *in situ* hybridisation

MLH1	MutL homolog 1
NRGene	Next Generation Complex Genome Assembly
PCR	Polymerase Chain Reaction
Pg	Paragon
PHR	Polymorphic high resolution
Ph1	Pairing homoeologous 1
PoPSEQ	Population Sequencing
QTL	Quantitative Trait Loci
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SNP	Single nucleotide polymorphism
SS DNA	Salmon Sperm DNA
Syn.	Synonymous
TGAC	The Genome Analysis Centre
USDA	United States Department of Agriculture
V/V	Volume to volume
WRC	Wheat Research Centre
WSMV	Wheat Streak Mosaic Virus

1. Introduction

1.1. The importance of wheat as a crop

The domestication of wheat over 10,000 years ago marked a pivotal moment in humanity's history, when human lifestyle began to shift away from nomadic hunters towards more settled communities. Simultaneously, human influence directly affected wheat as it underwent domestication. The earliest cultivated wheat varieties would have essentially been landraces; lines selected by farmers due to their yield or other advantageous traits such as disease resistance. Traits disadvantageous to agriculture, such as the shattering spike at maturity and hulled ears with tightly held glumes, were lost from these newly domesticated land races, in favour of the free-threshing, non-shattering ears (Shewry, 2009). This process, known as domestication syndrome, alongside two hybridisation events gave rise to the modern hexaploid wheat crop utilised today as a global food source, essential now for our global food security.

Wheat is an important source of carbohydrate and plant protein within the human diet, and when eaten as a whole grain contains multiple nutrients and high levels of dietary fibre. Since its domestication wheat has become an integral part of over 50% of the global population's diet, with 2.5 billion people consuming the crop in 89 countries every day (CGIAR, 2014). Wheat can also be used as an animal feed source, has multiple industrial uses and can be used as a biofuel source. In 2014, over 220 million hectares of wheat were harvested globally – an area greater than any other crop, including rice and maize (FAOSTAT, 2014). This achievement came from over 25,000 accessions of hexaploid wheat that were capable of being grown at an unrivalled range of latitudes and altitudes. In 2016, wheat overtook rice as the second most-produced crop on a global scale, with a record 749 million tonnes harvested (FAOSTAT, 2017). Even with these record breaking figures in recent years, global demand for wheat is increasing faster than yield, predominantly due to the persistently growing human population. This mismatch in demand and supply must be addressed in order to conserve global food security for future generations.

By 2050 the world population is predicted to exceed 9.8 billion people, rising to 11.2 billion by 2100 (Figure 1.1). A large proportion of this population

increase will come from a small number of developing countries, such as Nigeria, whose population is set to overtake that of the USA (United Nations, 2017). This rapid population increase will place tremendous pressure on world food production requiring an increase of 70%, or an additional 1 billion tonnes of cereal, simply to maintain our current global dietary standards – leaving at least 1 billion people still malnourished (Gustafson, Borlaug and Raven, 2010).

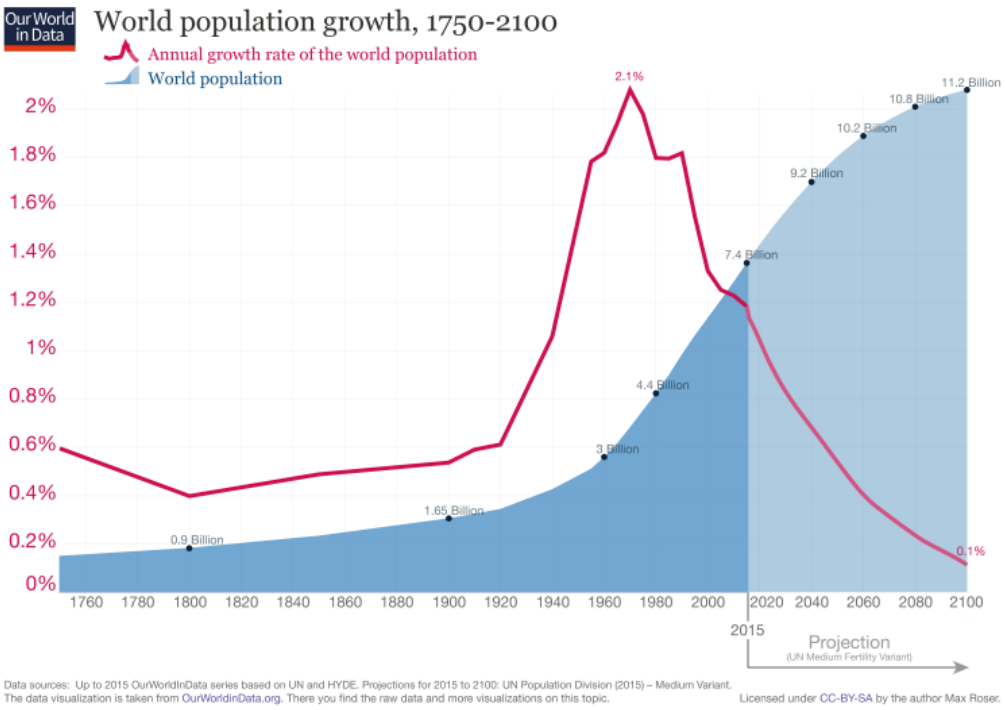


Figure 1.1: World population to date and predictions to 2100, with annual growth rate of the world population overlain. World population projection is the UN Medium Variant from the 2015 revision published by the UN Population Division.

An additional complication to the growing food crisis is urban expansion. Land suitable for farming is fast becoming a limited commodity. Any future increase in production capacity will need to be achieved on land that is already cultivated, as expanding towns and cities take precedence over farmland. Utilising unsuitable land for crops will not result in the increase in yields desired, and instead could exacerbate current environmental issues, such as the increasing droughts in Australia (Brisson *et al*. 2010).

Alongside the pressures of an increasing population and limited land availability, future wheat production faces other challenges. Climate change and intensive farming practices are two factors increasing the prevalence of drought, infertile soils, saline soils and disease levels in all countries

producing cereal crops, directly impacting yields. As a result of this, the demand for wheat varieties capable of sustained or improved performance in non-optimum conditions with increased adaptability is on the rise (Foulkes *et al.*, 2007). Increasing, or at least stabilising, wheat yields for future generations is arguably the most important challenge for agriculture in this millennium. Wheat is the staple crop of humanity, as both a direct nutrition source and animal feed crop. In order to understand how to improve wheat for the future, its evolution and subsequent domestication must be looked at.

1.2. The evolution of modern hexaploid wheat

Modern hexaploid bread wheat is a member of the Poaceae family, more commonly known as the grass family, which includes other important crop species such as rice and maize. Within this family, wheat is classified as a member of the Triticeae tribe, Triticum genus.

Approximately 150,000 years ago, the first hybridisation event between *Triticum urartu* (*T. urartu* - diploid A genome donor of polyploid wheat) and an extant or extinct *Aegilops* species (diploid B genome donor of polyploid wheat) gave rise to *Triticum turgidum* (*T. turgidum* - tetraploid, genome AABB). This initial hybrid then underwent natural amphiploidisation (Shewry, 2009).

During the Neolithic era (~ 10,000 years ago), the second hybridisation and subsequent amphiploidisation event occurred between *T. turgidum* and a wild grass, *Aegilops tauschii* (*Ae. tauschii* - diploid D genome donor of wheat), giving rise to the hexaploid wheat (*Triticum aestivum)* globally cultivated today(McFadden & Sears, 1946; Feldman, 1995). See Figure 1.2 for a summary of wheat evolution.

The two hybridisation events that gave rise to *Triticum aestivum* (*T. aestivum*) are hypothesised to have only occurred once, or at most, two times (Charmet, 2011). This monophyletic or diphyletic origin of wheat means that the available genetic diversity within the crops gene pool was limited by a severe genetic bottleneck. Further genetic erosion from the intensive farming practices placed upon wheat has further depleted the variation in the wheat gene pool. Any genetic variation within wheat that has arisen over the last 10,000 years can only be from genetic mutation or potential outcrossing with other species (King *et al.*, 2017).

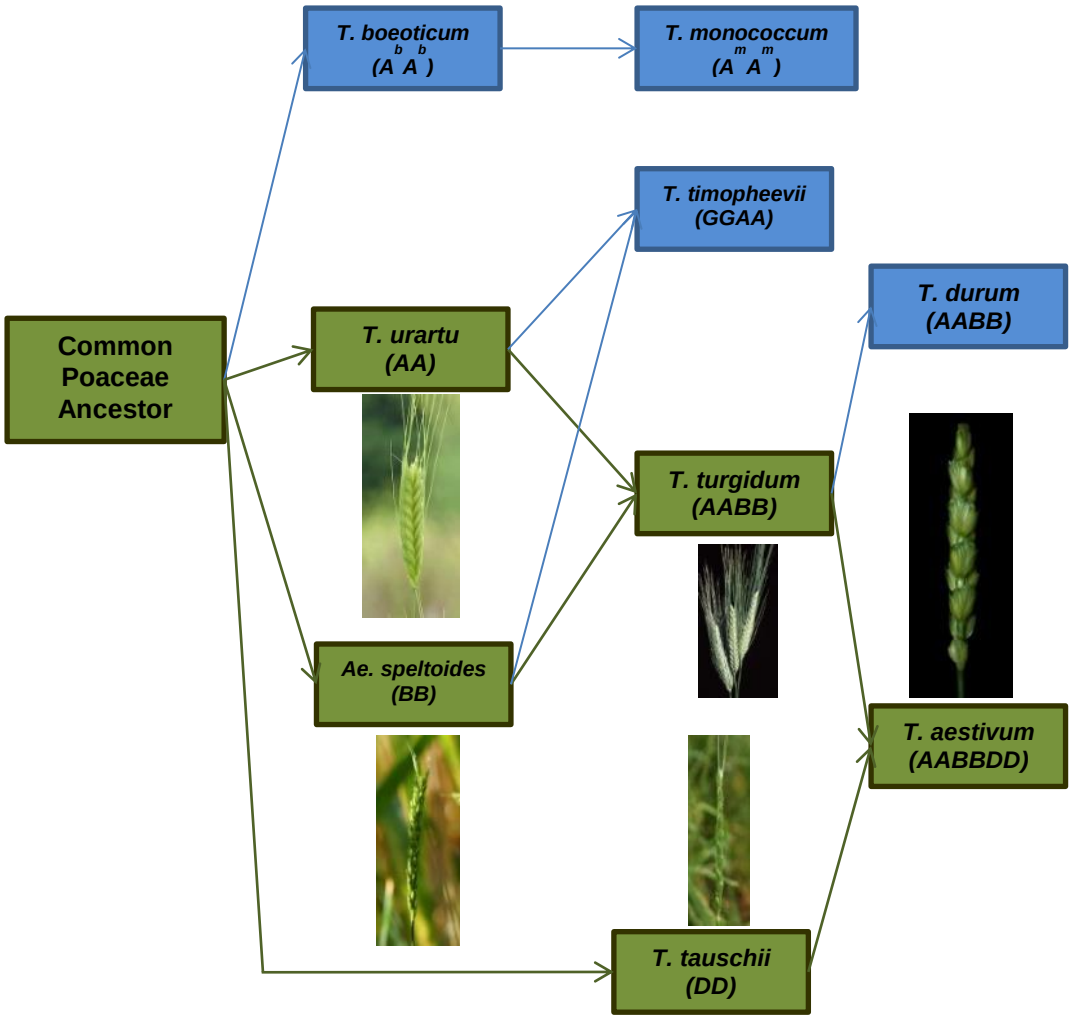


Figure 1.2: Diagrammatic representation of the evolution of *T. aestivum*. Species in blue boxes are related to wheat, but not directly involved with the evolution of modern hexaploid bread wheat. The species contained within green boxes are directly involved with the evolution of modern hexaploid bread wheat (*T. aestivum*).

The first hexaploid species of *T. aestivum* (2n=6x=42, AABBDD) cultivated were selectively bred by farmers to further improve its agronomic performance. This practice of selective breeding and the genetic bottleneck wheat underwent during its evolution, make wheat a genetically narrow crop species. Such species have already proven to be a risk to global food security, once with the Irish Potato Blight and more recently with the Banana Panama Disease. The risk they pose is due to the lack of variation in genetics making them susceptible to disease (Pingali & Heisey, 1999). When looking more specifically at wheat, Ug99, an accession of *Puccinia graminis* (wheat stem rust) was of cause for concern in the last decade, with up to 90% of the world's wheat cultivars considered susceptible (Singh *et al.*, 2011). Losses of up to 100% were recorded in some African fields. This accession was of

particular concern thanks to its ability to overcome resistance genes (Sr genes) that had protected wheat from other strains of the Black Stem Rust for decades (Singh *et al.*, 2015). In this scenario, disease resistance from wild relatives of wheat such as *Ae. tauschii* and *Ae. searsii* helped bring the outbreak back under control (Liu *et al.*, 2011; Olson *et al.*, 2013).

Spontaneous mutations were thought to be the only way that genetic diversity could have been introduced to the wheat gene pool after the two hybridisation events that led to its evolution. However, more recent studies have shown the gene flow between wheat and its wild relatives is possible, especially when the species are grown in polygenic fields. More surprisingly, wheat has been shown to have introgressions from rye in its gene pool, suggesting that spontaneous introgressions with wild relatives is possible, even with completely unrelated genomes (Ribeiro-Carvalho *et al.*, 1997). The number of occasions that this phenomenon has occurred is likely to be very low, due to the sterility issues that such hybrids face. However, recent research done using advanced SNP marker analysis has found more genetic diversity exists between different wheat accessions than previously known (Montenegro *et al.*, 2017). The question posed now is can we further increase the genetic diversity available for breeders to utilise in the wheat gene pool?

1.3. The history of wheat yields

Over the last half a century, global yield of wheat has increased steadily, despite the fact that the area harvested has overall been declining over the last 30 years (see Figure 1.3 and Figure 1.4).

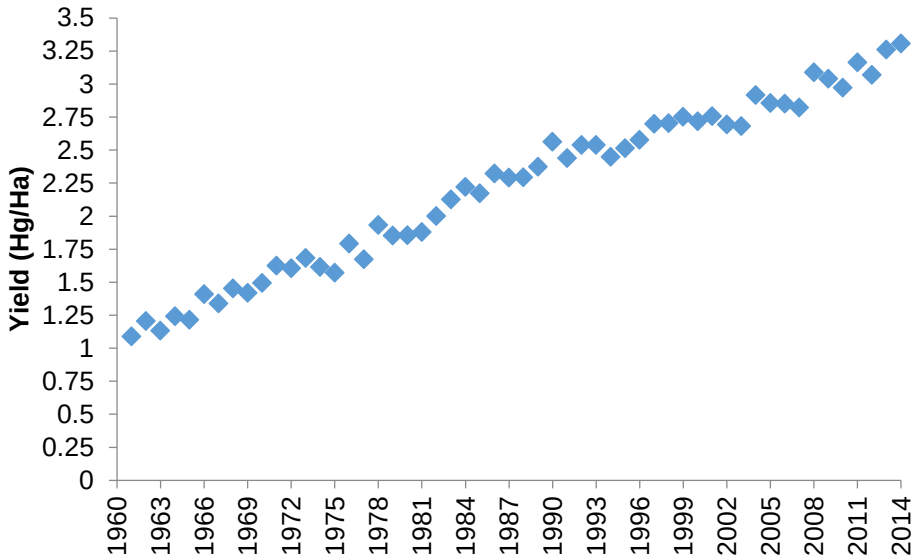


Figure 1.3: Global yield of wheat (hectograms/hectare) from 1960 to 2014 inclusive (FAOSTAT, 2014).

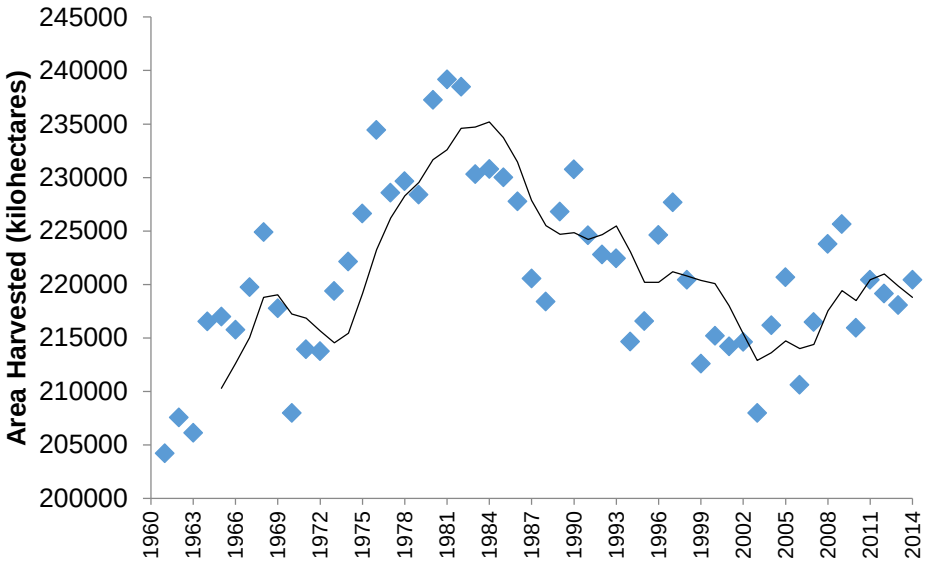


Figure 1.4: Area of wheat harvested globally in kilohectares from 1960 to 2014 inclusive. 5 year moving average included to visualise the trend in data (FAOSTAT, 2014)

Whilst the area being used for wheat production has stabilised over the last 20 years, yield has steadily increased. These gains can be attributed in part to genetic improvements introduced by the effort of breeders to improve the harvest index of wheat, which is the ratio of yield versus total crop biomass.

It is generally agreed that any further improvements in Harvest Index are limited in elite wheat cultivars as breeders have exhausted all genetic variation possible and the plants still need leaves, roots and stalks to actually function (Curtis & Halford, 2014). The use of inputs, such as fertilisers, has also played a role in achieving these steady yield increases by removing some environmental limits. However, it must be noted that due to the growing population pressure, increased legislation on the use of fertilisers and an increase in consumption *per capita,* yield increases are currently not enough to meet future demand (Curtis & Halford, 2014). This mismatch in supply and demand can partly be attributed to the genetic bottleneck that modern cultivars of wheat are under. Wheat's monophyletic/diphyletic evolution, combined with 10,000 years of selective breeding, has steadily depleted the genetic variability available for breeders to improve modern elite cultivars. Genetic improvements are still being made in wheat, however these improvements are often not fully seen thanks to the negative impacts of climate change upon cereal yields, e.g. high temperatures during grain filling. By 2050, it is predicted that the global demand for wheat will be around 900 million tonnes (Charmet, 2011). To achieve this volume, annual global increases in yield would need to be around 2.4%, yet for the last decade have averaged 0.9% (Ray *et al.*, 2013) - in Europe the rate of yield increases has actually steadily plateaued to around 0.1%. With these figures it becomes more apparent that other sources of genetic diversity, to help develop superior wheat varieties capable of tolerating climate change whilst providing increased yields, must be identified and utilised.

1.4. Increasing diversity in wheat

The genetic bottleneck caused by wheat's evolution, and the subsequent repeated use of elite cultivars being farmed under intensive selection for advantageous agronomic traits has depleted the gene pool of wheat of genetic diversity, contributing to the stagnation in genetic improvements (Feuillet, Langridge and Waugh, 2008).

In order to effectively increase the diversity available in wheat, the gene pools must be understood. The gene pool concept was first introduced in 1971 to aid the classification of both cultivated and uncultivated plants (Harlan and de Wet, 1971). *Triticeae* species can be separated into three gene pools that represent the potential genetic transfers for wheat improvement over short or

long term time frames. The primary gene pool species can be thought of as the homologous gene pool, which contains species with the same genomes as wheat. The primary gene pool includes the hexaploid landraces, cultivated tetraploids and the diploid donors of the A and D genomes to durum and bread wheat (Feuillet, Langridge and Waugh, 2008).

The secondary gene pool is partially homologous; it contains species with at least one of the common wheat genomes. It includes the mostly polyploid *Triticum* and *Aegilops* species as well as the diploid species of the *Sitopsis* section. Gene transfers in the primary and secondary pools occur as a consequence of direct crosses, breeding protocols, homologous exchange between the related genome or through use of special manipulation strategies among the non-homologous genome.

Diploid and polyploid species with non-homologous genomes are members of the tertiary gene pool. Due to the lack of a common genome with wheat, genetic transfers from the tertiary gene pool into a wheat background require assistance to enable homoeologous exchanges. A summary of the gene pools and some example species within each are shown in Figure 1.5.

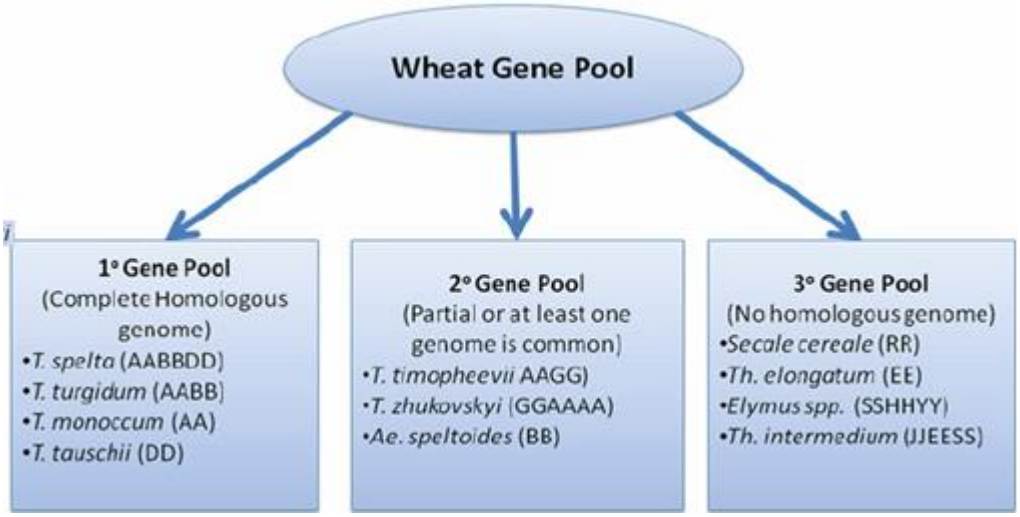


Figure 1.5: Diagrammatic representation of the primary, secondary and tertiary gene pools of wheat with example species in each (Chaudary *et al.*, 2014)**.**

Increasing the genetic diversity within the gene pool of wheat would give breeders new variation in elite cultivar lines to introduce a plethora of traits, including increased yield potential and improved disease tolerances. Multiple approaches have been undertaken to achieve this, including introgressing variation from land races, synthetic cultivars and wild relatives of wheat.

1.4.1. Land races

Land races are early cultivated varieties of wheat that are adapted to their local environment. Due to the large range of latitudes and altitudes that wheat can be cultivated within there is considerable diversity within landrace collections, as each landrace contains unique adaptations to suit its place of origin. The landraces, *T. turgidum* and *Ae. tauschii* are in the primary gene pool of wheat; this means they easily breed with wheat as their genomes are homologous. Therefore, introducing diversity from direct crosses with these species is an attractive breeding strategy.

The landraces were initially recognised as a potential source of genetic improvement during the late 1960s and efforts to identify and characterise valuable genes in landraces has subsequently increased (Zohary, Harlan and Vardi, 1969; Wingen *et al.*, 2017). Examples of previous successes achieved with landrace gene transfer into common wheat include resistance to stem rust, leaf rust, powdery mildew and tan spot from *T. turgidum* ssp. *durum* and from emmer wheat (*T. turgidum* ssp. *dicoccum*) (Cox, 1998).

The decrease in sequencing costs over the last decade has enabled sequencing to become routinely used in breeding programmes and can also be used to help identify and capture variation in landrace collections (Lopes *et al.*, 2015). Whilst the attractiveness of the chromosome homology makes the landraces seem like an obvious choice to improve wheat gene pool diversity, the diversity present is not as varied as can be found within the wild relatives of wheat.

1.4.2. Synthetics

Synthetics are an alternative approach to increasing wheat genetic diversity. The two hybridisation events that gave rise to *T. aestivum* involved very few individual plants. Other individual plants from the same species that hybridised to become hexaploid bread wheat could contain genetic variation not currently available in wheat. Synthetics were designed to try and incorporate any such variation by ‘recreating’ the evolution of bread wheat. In most cases, *T. turgidum* ssp. *durum* was used as the AABB donor and crossed with *Ae. tauschii*, the DD genome donor. Today, there are over 1,000 different synthetic wheats and the practise of generating and investigating synthetics as a source of genetic improvement in wheat is well established (Mujeeb-kazi, Rosas and Roldan, 1996; van Ginkel and Ogbonnaya, 2007).

The potential advantages of using synthetics to increase the genetic variation in the wheat gene pool are made apparent by Table 1.1.

Table 1.1: Previous successes of diverse traits identified in synthetic lines by CIMMYT. Table taken from (van Ginkel and Ogbonnaya, 2007).

Trait	Reference
Agronomic features	
• **Yield under irrigated conditions**	• Villareal *et al*. (1994a)
• **Yield under rain fed drought conditions**	• Reynolds *et al*. (1999)
• **High thousand kernel weight**	• Calderini & Reynolds (2000)
• **High above ground biomass**	• Villareal *et al*. (1994a)
• **High photosynthetic rate**	• Blanco *et al*. (2000)
• **Other morphological traits**	• Villareal *et al* (1994)
Abiotic tolerances	
• **Tolerance to drought**	• Reynolds *et al*. (1999)
• **Tolerance to frost at flowering**	• Maes *et al*. (2001)
• **Tolerance to salinity**	• Schachtman *et al*. (1992)
• **Tolerance to waterlogging**	• Villareal *et al*. (2001)
Biotic resistances	
• **Stripe rust (*Puccinia striiformis*)**	• Assefa & Fehrmann (2000)
• **Leaf rust (*Puccinia recondite*)**	• Ma *et al*. (1995)
• **Stem rust (*Puccinia graminis*)**	• Marais *et al*. (1994)
• **Leaf blotch (*Septoria tritici*)**	• Arraiano *et al*. (2001)
• **Glume blotch (*Septoria nodorum*)**	• Loughman *et al*. (2001)
• **Crown rot *(Fusarium graminearum*)**	• Mujeeb-Kazi *et al*. (2001)
• **Yellow leaf spot (*Pyrenophora tritici-repentis*)**	• Cox *et al*. (1992)
• **Leaf blight (*Helminthosporium*)**	• Mujeeb-Kazi *et al*. (2001)
• **Powdery mildew (*Erysiphe graminis*)**	• Kong *et al*. (1999)
• **Karnal blunt (*Neovossia indica*)**	• Villareal *et al*. (1994b)
• **Cereal cyst nematodes (*Heterodera acenae*)**	• Eastwood *et al*. (1991)
• **Root lesion nematodes (*Pratylenchus spp.*)**	• Thompson *et al*. (1999)
• **Greenbug (*Schizaphis graminum*)**	• Hollenhorst & Joppa (1983)
• **Hessian Fly (*Mayetiola destructor*)**	• Tyler & Hatchett (1983)
Quality features	
• **Proteins**	• William *et al*. (1993)
• **Glutenins**	• Pfluger *et al*. (2001)
• **Zinc efficiency**	• Cakmak *et al*. (1999)

In addition, because the genomes within the synthetics are homologous with those in bread wheat, hybrids created between synthetics and elite cultivars should have a reduced detrimental impact on their fertility, although problems can arise during chromosomal doubling. Similarly to using landraces, this approach only utilises variation within the primary gene pool of wheat. Whilst this may be an easier approach than introducing variation from the wild relatives of wheat, it does not have the potential variation that the tertiary gene pool of wheat offers.

1.4.3. The wild relatives of wheat

The third option for improving the genetic variation within the wheat genome is to outbreed with wild relatives of wheat found in the tertiary gene pool. The landrace, synthetics and wild relatives such as *T. timopheevii* are more easily crossed to modern hexaploid wheat due to their homologous or semi-homologous genomes. Most wild relatives of wheat are in the tertiary gene pool and are more challenging to breed successfully with wheat. This is because species within the tertiary gene pool have no genomes homologous with modern bread wheat and the chromosome ploidy can vary from diploid to decaploid. The tertiary gene pool consists of hundreds of other Triticeae species more distantly related to wheat; therefore it is within this gene pool that the highest potential for introducing variation into wheat can be found. This gene pool includes *Thinopyrum elongatum.* Wide hybridisation between wheat and species within the tertiary gene pool would broaden the germplasm base the greatest, however issues such as early embryo abortion and hybrid seed inviability usually limit success. Previous methods that have been used to overcome the reproductive barrier between wheat and its wild relatives include: colchicine treatment, using a genetic 'bridge' such as wheat addition or translocation lines, radiation, gametocidal chromosomes or *ph1* deletion mutants. Each method is explained below.

When an initial cross with a wild relative of wheat is carried out, the F_1 hybrids formed are amphihaploid. Theoretically, due to the lack of homologues, no chromosome pairing should occur within the gametes and the plant would most likely display complete sterility. To overcome this barrier, colchicine treatment can be used on immature embryos to force chromosomal doubling, producing an amphidiploid now capable of producing viable gametes and restoring fertility. One negative of this approach is that the insertion of a whole

wild relative genome into a wheat background results in negative phenotypic traits being transferred alongside potentially beneficial ones (Qi *et al.*, 2007).

An alternative approach to overcome the reproductive barrier is to transfer genetic variation from wild relatives to wheat via addition, substitution, translocation and introgression lines (Figure 1.6). A wheat addition line has additional chromosomes in comparison to the wild type. For example, in a wheat disomic addition line the chromosomal number will be 2 higher than normal; 44 rather than the usual 42. In contrast, a wheat substitution line has the usual 42 chromosomes, but at least one of the wheat chromosomes will have been replaced by a chromosome from another species. A translocation line is similar to a substitution line except that only one arm of a chromosome will have been replaced by the homoeologous region from a wild relative genome. An introgression line is formed by a cross-over event within the chromosome arm.

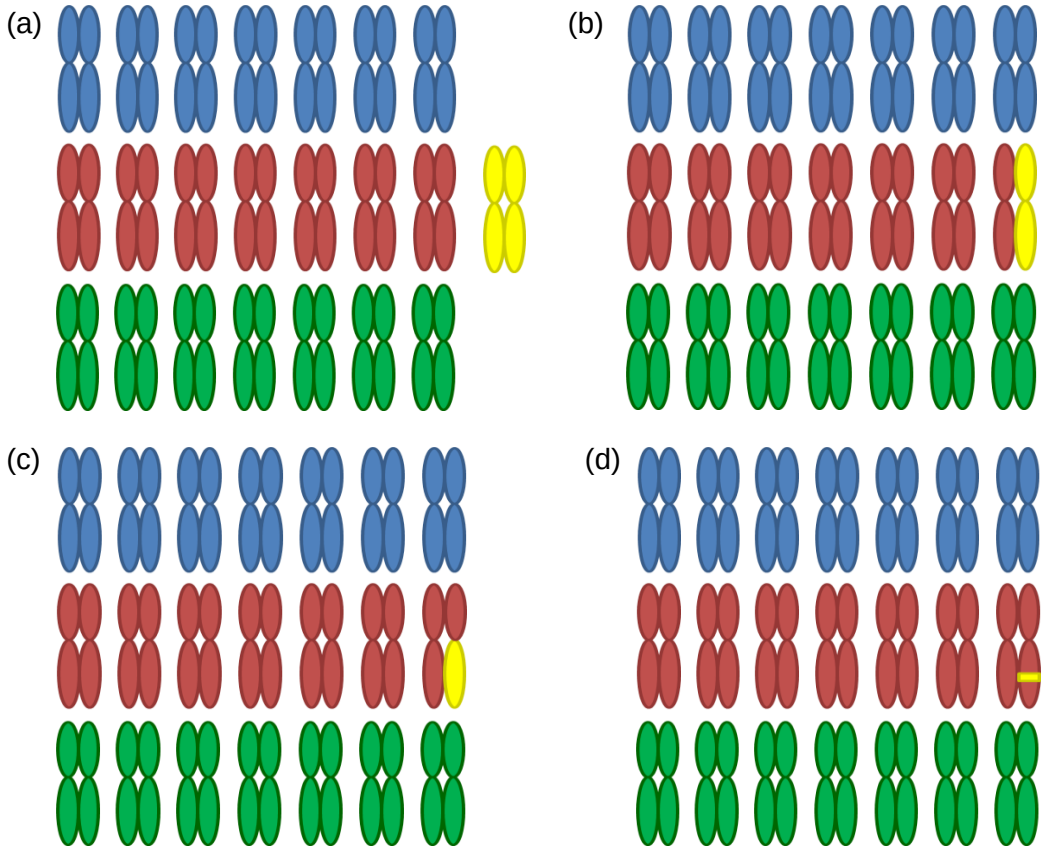


Figure 1.6: Representation of (a) a disomic addition line, (b) a monosomic substitution line, (c) a Robertsonian translocation line and (d) an introgression line all in a wheat background. Blue represents the A genome, red represents the B genome and green represents the D genome of wheat. The yellow represents genetic material from another genome, for example the E genome from *Th. elongatum* 401007.

Addition lines have proven difficult to maintain, as a wild relative addition line does not have a balanced genotype and loss of the wild relative chromosomes is more likely to occur under pollen selection. Substitution lines do not have the problem of producing an unbalanced genotype as the wild relative chromosome physically replaces the homoeologous wheat chromosome. However, the transfer of such a large section of wild relative chromatin can have a negative impact via linkage drag. The same issue can be seen with translocation lines. Therefore, introgression lines are the best option for introducing variation from a wild relative of wheat with limited linkage drag.

The development of cytogenetic and chromosomal engineering techniques allowed different methods to be used to introduce genetic variation from wild relatives of wheat. Irradiation, gametocidal genes and induced homoeologous pairing could all be used to produce recombinant chromosomes with targeted genes to be bred into a wheat background (Sears, 1981).

Radiation induces random chromosome breakage and translocations. Therefore, achieving transfer of targeted genes is a challenge and would need to be confirmed in the offspring's phenotype. The size of the translocation produced would also be random (Sears & Gustafson, 1992). As the radiation induces random chromosome breakage, there is a chance that segments transferred will not be from the equivalent segment of wheat they are replacing, resulting in possible loss of function.

Gametocidal chromosomes (Gc), sometimes known as 'selfish' chromosomes, ensure they are passed on to the following generation by causing sterility in gametes not containing them through chromosome breakage. This chromosomal breakage has been used to produce wheat-wild relative translocation lines. Whilst being safer to handle than mutagens or radiation, the breakages induced by Gc systems are still random (Endo, 2007).

The Nottingham BBSRC wheat breeding programme used wheat with the *ph1* deletion mutation for the initial cross with wild relatives to enable homoeologous recombination in the gametes of F_1 hybrids. Induced homoeologous pairing became possible with the discovery of the *Ph1* (pairing homoeologous 1) locus in the late 1950's (Sears & Okamoto, 1958). The *ph1* deletion mutants were shown to allow homoeologous chromosomes to pair,

enabling direct transfer of genetic variation between two unrelated species. A more detailed account of the role of the *Ph1* gene is in section 1.5.

There are numerous successes of transferring genes into a wheat background from a wild relative using induced homoeologous recombination. The most common transference is of disease resistance genes (see Table 1.2).

Table 1.2: Examples of previous traits or genes transferred from wild relatives of wheat (focussing on species within the WISP breeding programme) to *T. aestivum* via homoeologous recombination.

Wild Relative	Gene Transferred (if identified)	Trait	Reference
Aegilops caudata	*LrAC*	Leaf rust resistance	(Riar *et al.*, 2012)
Aegilops searsii	-	Ug99 resistance	(Liu *et al.*, 2011)
	Lr66	Leaf rust resistance	(Marais *et al.*, 2009)
Aegilops speltoides	*Pm53*	Powdery mildew resistance	(Petersen *et al.*, 2014)
Amblyopyrum muticum	-	Powdery mildew resistance	(Eser, 1998)
Secale cereale	*Sr50*	Ug99 resistance	(Singh *et al.*, 2015)
Thinopyrum bessarabicum	-	Salt tolerance	(King *et al.*, 1997)
Thinopyrum elongatum	*Lr19*	Leaf rust resistance	(Zhang *et al*. 2005)
	Fhb-7EL	Fusarium head blight resistance	(Ceoloni *et al.*, 2017)
	Wsm3	Wheat streak mosaic virus resistance	(Danilova *et al.*, 2017)
Thinopyrum intermedium	*Bdv2, Bdv3, Bdv4*	Barley yellow dwarf virus resistance	(Ayala-Navarrete *et al.*, 2007)
Triticum tauschii	*Lr21, Lr40, Lr41 Lr42*	Leaf rust resistance	(Cox, Raupp and Gill, 1993)
	H26	Hessian Fly resistance	(Wang *et al.*, 2006)
Triticum timopheevii	*Pm27*	Powdery mildew resistance	(Jarve *et al.*, 2000)
Triticum urartu	*PmU*	Powdery mildew resistance	(Qiu *et al.*, 2005)

1.5. Meiosis and the *Ph1* gene

From the moment humans began to domesticate wheat, variation within farmers' fields was scrutinised and the most agronomically favourable traits were continued forwards by breeding. This variation within the fields was present due to meiosis. Meiosis is a specialised type of cell division that results in the production of four, non-genetically identical cells from one parent cell. Its primary function is to produce gametes with half the somatic number of chromosomes to ensure chromosome number does not double with each generation, whilst enabling genetic variation to exist within a species.

As knowledge of DNA and genes advanced, the *Ph1* locus was discovered in wheat, posing a problem for breeders who wished to exploit genetic variation in distant relatives of wheat. The problem was that the presence of *Ph1* gene was shown to prevent homoeologous chromosomes from recombining (Riley & Chapman, 1958; Sears & Okamoto, 1958), thereby preventing any variation from wild relatives of wheat being introduced into a wheat background via a direct cross.

Molecular characterisation of the *Ph1* locus identified its location to be on the long arm of chromosome 5B, approximately 1.0cM from the centromere (Wall, Riley and Gale, 1971). More recent studies using deletion lines have further localised the *Ph1* locus to a 2.5Mb region of the 5B wheat chromosome which contains a cluster of *cdc2*-related genes (Griffiths *et al.*, 2006). This location seems likely to contain the *Ph1* candidate gene, as detailed bioinformatics have shown the *cdc2*-related cluster in wheat is highly similar to *Cdk2* kinase; a kinase essential for meiosis in humans (Al-Kaff *et al.*, 2008).

Wheat is a hexaploid organism, yet during meiosis it behaves cytologically as a diploid (Riley & Chapman, 1958). This diploid behaviour is predominately down to the action of the *Ph1* gene. The *Ph1* gene arose during polyploidisation and only allows homologous chromosomes to recombine during meiosis. This means that chromosome 1A can only pair with chromosome 1A, 1B with 1B and so on. 1B cannot pair with 1D. It limits the chromosomes to recombining with their true pair (Sears, 1976). Figure 1.7 provides a visual explanation of the difference between homologues and homoeologues.

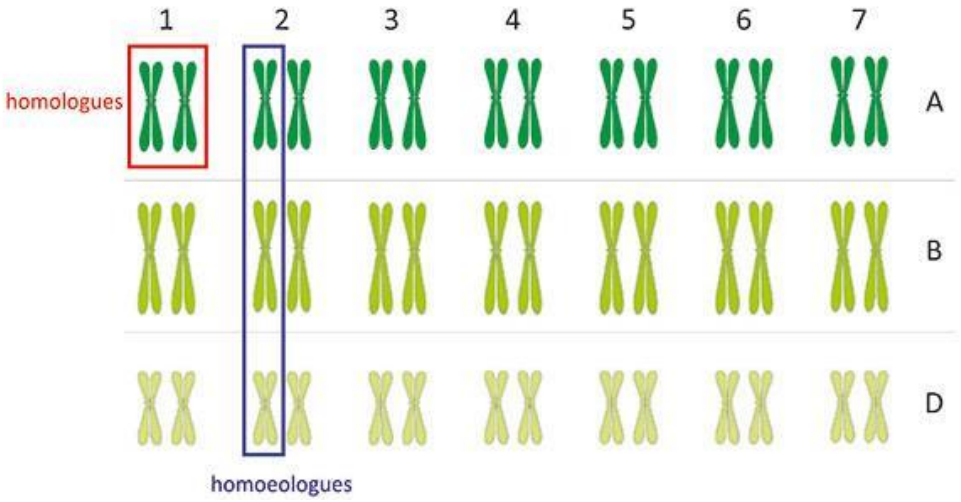


Figure 1.7: Diagrammatic representation of the hexaploid wheat genome separated into its three genomes A, B and D. The red box highlights homologous chromosomes, whereas the blue box highlights homoeologous chromosomes (Moore, 2014).

In order to fully utilise the *Ph1* gene for breeding with wild relatives of wheat extensive research has been done over the last 60 years into its mode of action. The name *Pairing homologous 1* was initially used as it was assumed that the locus directly inhibited the pairing of homoeologous chromosomes. However, this theory can be considered disproved as studies with wheat and wild relative hybrids where no homologues were present showed homoeologous chromosome pairing in the presence or absence of the *Ph1* locus. Similar studies also showed that the *Ph1* locus did not prevent synaptonemal complexes forming between homoeologues (Gillies, 1987; Moore, 2014).

The most recent proposed mode of action for the *Ph1* locus is the promotion of homologue pairing, rather than the prevention of homoeologous pairing. Therefore, it increases the stringency of pairing homologous chromosomes or resolves incorrect homoeologue associations during meiosis. Recent studies have utilised immunolabelling of the protein MLH1. MLH1 is required for the resolution of Double Holliday Junctions during cross-overs in meiosis and is activated by *Cdk2*-type activity (Martín *et al.*, 2014). Studies with wheat-rye hybrids showed that *Ph1* locus prevented the resolution of Double Holliday Junctions by reducing the level of *Cdk2*-type activity, thereby inhibiting the MLH1 gene and suppressing recombination between homoeologues (Greer *et al.*, 2012).

The ability of *Ph1* to impact homoeologous recombination has made it an obstacle for breeders to overcome in order to introduce variation from wild relatives of wheat. There have been numerous notable previous successes, with genetic variation being introduced from many members of the *Thinopyrum* genus, including *Th. ponticum, Th. elongatum* and *Th. bessarabicum* (King *et al.*, 1997; Shen, Kong and Ohm, 2004; Oliver *et al.*, 2006).

The genetic control of meiotic chromosome pairing in hexaploid *T. aestivum* was first demonstrated in the cultivar Chinese Spring (Okamoto, 1957). This led to the development of the first *Ph1* deletion lines in the late 1950s and enabled homoeologous chromosomes to recombine, meaning chromosome 1A could pair with 1B, 1D, or a homoeologue from a wild relative (Sears and Okamoto, 1958). This discovery enabled researchers to directly transfer genetic variation from the tertiary gene pool into wheat. The F_1 hybrids produced from crosses between a *ph1* deletion mutant and a wild relative would essentially be haploid, containing one complete genome from each parental (See Chapter 4 – Germplasm Development). Therefore, these lines will also still contain the *ph1* deletion from the wheat parent. This means that during gametogenesis, homoeologous recombination is forced, as no homologous genomes are present and the *Ph1* gene cannot correct any incorrect associations.

Okadaic acid has been shown to mimic the effects of *Ph1* deletion and therefore could be used to temporarily 'switch off' the *Ph1* locus, without the need to actually delete the locus, enabling homoeologous recombination (Knight *et al.*, 2010). Interestingly, in the absence of *Ph1,* altered environmental conditions have been shown to increase the number of MLH1 sites, genetic locations known to be essential in cross-over events, in both homoeologues and homologues (Martin *et al.*, 2017). Future studies based on these discoveries will allow breeding programmes to take advantage of the large number of unexplored genes in wild relatives of wheat in exciting and more efficient ways.

The breeding programme at the Nottingham BBSRC Wheat Research Centre (WRC) used *ph1* deletion mutants in a Chinese Spring background to make the initial cross with *Th. elongatum* 401007. All future back-crossing of the hybrids produced used wild type wheat with the *Ph1* locus restored to help restabilise the genetics of each line.

1.6. An overview of *Thinopyrum elongatum*

This section briefly introduces the wild relative used within this research, *Th. elongatum* 401007. A more in depth introduction is included in the beginning of the Germplasm Development chapter (see section 4.1).

Thinopyrum elongatum (*Th. elongatum*) is a member of the Eurasian *Thinopyrum* genus. In published work, it is most frequently described as a diploid (2n = 2x = 14, EE), however other research has shown the species also contains tetraploid, hexaploid and decaploid accessions (Mao *et al.*, 2010; Chen *et al.*, 2013; Guo *et al.*, 2016). *Th. elongatum* is a wild relative of wheat with a non-homologous genome in comparison to wheat, and so is classified within wheats tertiary gene pool. This means that variation from *Th. elongatum* 401007 cannot be introduced into wild type wheat via a direct cross due to reproductive barriers. As previously stated, the Nottingham BBSRC wheat breeding programme used a wheat *ph1* deletion mutant for the initial cross with *Th. elongatum* 401007 to enable homoeologous recombination and overcome this reproductive barrier.

Th. elongatum contains genes for many favourable agronomic traits, including an abundance of disease tolerances, some of which have already been exploited within wheat breeding. For example, the *Lr19* gene was transferred into wheat from *Th. ponticum* and conferred rust resistance (Sharma & Knott, 1966). In addition to rust resistance, the *Lr19* gene has since been linked to an increase in yield, even in the absence of rust, and an increase in biomass and seed number (Reynolds *et al.*, 2001). Other agronomic traits transferred to wheat from *Thinopyrum* species include: Barley Yellow Dwarf Virus (BYDV) resistance, salt tolerance, reduced height, Wheat Streak Mosaic Virus (WSMV) resistance, Fusarium Head Blight (FHB) resistance and Common Root Rot resistance (Li & Wang, 2009; Chen *et al*. 2012; Chen *et al*. 1998; Zhang *et al*. 1996; King *et al*. 1997; Suiyun *et al*. 2004).

Despite its fairly prolific use as a source of genetic variation for wheat, the genus has been particularly difficult for taxonomists to define, thanks in part to the amount of polyploidisation that occurred within the group during its evolution and the close genetic relationship between the genomes that are present within the genus. Previous classifications have seen *Thinopyrum* species clustered together with *Elytrigia*, *Lophopyrum* and *Agropyron* geneses, as they show remarkable similarities in their physiology. Even

though *Thinopyrum* has been classified as a separate genus since the late 20th century, the nomenclature of *Thinopyrum* species is still highly variable in previously published research. For example, *Thinopyrum ponticum* (Podp.) (2n=10x=70, $JJJJJJJ^{St}J^{St}J^{St}J^{St}$) has been synonymous to *Elytrigia elongata* (Host) Nevski, *Agropyron elongatum* (Host) P.Beauv and *Lophopyrum elongatum* (Host) A.Love (USDA, 2014). This means that when assessing previous literature, it is essential to check the ploidy and genomic composition of the plant the work is referring to where possible before making comparisons.

Two diploid species within the *Thinopyrum* genus are *Th. bessarabicum* (2n = 2x = 14, JJ) and *Th. elongatum* (2n = 2x = 14, EE). The E and J genomes have been shown to be closely related, with some studies suggesting that they are actually the same genome, just modified versions of each other, and therefore should be reclassified as one (Wang, 1985; Wang & Hsiao, 1989). An excellent literature review is available on past studies of the genomic relationships between the J and E genomes, but the verdict on whether the genomes are synonymous or distinct is not currently conclusive (Wang & Lu, 2014).

The genomic constitutions of the polyploids within the *Thinopyrum* genus become even more complicated than the diploids. The issue of the E or J genome is still present, along with genomes from other progenitor species. The St genome from a *Pseudoroegneria* progenitor is present in the polyploids of the *Thinopyrum* genus, however it varies between species (Wang, 2011). Within the hexaploid *Th. intermedium* (2n = 6x = 42, $JJJ^{St}J^{St}StSt$) whole St chromosomes can be distinguished using genomic *in situ* hybridisation (GISH) as well as modified J^{St} chromosomes, where the St genome is localised to the centromeric region, whereas in *Th. ponticum* (2n = 10x = 70, $JJJJJJJ^{St}J^{St}J^{St}J^{St}$) the St genome is only present as part of the modified J^{St} chromosome (Chen, Conner, Laroche, *et al.*, 1998). Further complication has recently arisen by evidence of the V genome, from *Dasypyrum villosum,* being capable of hybridisation with centromeric regions of chromosomes within *Th. intermedium* (Mahelka, Kopecký and Paštová, 2011; Wang *et al.*, 2015). Essentially, whilst the *Thinopyrum* species are known to contain many advantageous traits for wheat genetic improvement, their complicated genetics could prove a challenge to work with for researchers and breeders alike.

Focussing on *Th. elongatum*, previous cytogenetic studies have suggested the species can have diploid (2n = 2x = 14, EE), tetraploid, hexaploid and decaploid accessions (Mao *et al.*, 2010; Chen *et al.*, 2013; Guo *et al.*, 2016), although there is still uncertainty as to whether the decaploid accessions should be re-classified as *Th. ponticum.*

Chromosomal spreads of the accession used within this research, *Th. elongatum* 401007*,* have repeatedly shown 72 chromosomes making the 401007 accession a decaploid with aneuploidy. The seeds for this research were obtained from the USDA and collected before the 1970's. Due to advances in genetic markers and alterations in the *Thinopyrum* taxonomy classification since these seeds were collected in the field, it is important to bear in mind that the *Th. elongatum* 401007 accession obtained from the seed bank may actually be *Thinopyrum ponticum*.

Potential uses of *Thinopyrum* genetic variation for wheat improvement include: various rust resistances (Friebe *et al.*, 1996), drought tolerance (Lammer *et al.*, 2004), salt tolerance (Colmer, Flowers and Munns, 2006), waterlogging tolerance (McDonald *et al.*, 2001) and potential improved photosynthetic capacity from an improved source to sink ratio (Reynolds *et al.*, 2001). *Th. elongatum* is also perennial, which may confer advantages in terms of nutrient use efficiency and improved soil stability (Lammer *et al.*, 2004; Murphy *et al.*, 2010). Potential negative agronomic traits associated with *Th. elongatum* include its increased height, a reduced number of spikelets per ear and the seeds are not free-threshing. The perennial nature of the wild relative also infers a longer growth habit within the hybrid lines. They also need longer in vernalisation, take longer to reach sexual maturity and longer to set seed – traits not desirable in modern agricultural practices.

Whilst the genomic constitution of *Th. elongatum* presents certain challenges to using it in a breeding programme, the vast amount of potential variation in the gene pool and the apparent ease at which it transfers agronomic traits of interest into a wheat background makes it of considerable interest to wheat breeders.

1.7. Detection and characterisation of introgressions

1.7.1. The introduction and utilisation of DNA markers for breeding programmes

Genetic markers are known sequences or landmarks on chromosomes that can be used as a reference for other genes of interest, to differentiate between individual plants or to construct genetic maps. Prior to the development of molecular markers, crop breeding programmes relied on morphological markers that were visible in a phenotype, such as seed shape or flower colour. However, such methods had their drawbacks: morphological markers are limited in their number and often are only manifested in adult phenotypes. They can also be subject to impact from gene interactions and environmental influences. Expert knowledge may be required to recognise the traits, making methods that utilise morphological markers potentially labour intensive, expensive and inefficient at introducing quantitative traits into crops. Finally, morphological markers may result in most of the variation present from a species being ignored, as it is not visible in a phenotype.

Advances in molecular science brought around the use of molecular markers to plant breeding. Molecular markers enabled researchers to detect polymorphisms at the sub-cellular level and were quickly put to use in a variety of projects including: characterising germplasm, developing linkage maps, identifying markers associated with QTLs and rapid introgression of simply inherited traits. The advantages molecular markers brought to research were numerous and included: not needing an adult phenotype for material to be assayed, being theoretically limitless in number, being unaffected by the environment and usually located in non-coding regions of DNA, making them selectively neutral. Variation across an entire genome could also now be theoretically harnessed, rather than just the gene affecting phenotypes.

Today, there are many different types of molecular markers available, which will be briefly summarised in the following section. When choosing a molecular marker system to utilise for research, the following characteristics must be considered:

- Marker mutation rate
- Abundance and distribution across the genome
- Degree of polymorphism
- Selective neutrality
- Accuracy
- Reproducibility
- Cost
- Automation potential (high-throughput capabilities)

Ideally, markers would have low mutation rate, high abundance and good distribution across the entire genome, high degree of polymorphism, be selectively neutral, easy to isolate, highly reproducible, low cost and suit automation potential. In reality, each marker system has its own advantages and disadvantages and must be selected based on research requirements.

The first molecular marker system developed was chemical assays for isozymes. These enzyme-based assays did not detect variations in the DNA, but in the protein products. This limited their use and their uneven distribution across the genetic map made them quickly redundant (Singh & Singh, 2015). Restriction endonucleases were then discovered from work on bacteria in the 1950s (Luria & Human, 1952). Their ability to cleave DNA sequences at specific locations known as restriction sites, and the subsequent visualisation of the restriction fragments produced using Southern blotting, led to the development of restriction fragment length polymorphisms (RFLPs). There were many advantages to these markers, firstly, they could detect variation in both coding and non-coding regions of DNA and the theoretical number of RFLPs available was limitless. In reality, the number of markers was inhibited by the availability of suitable hybridisation probes and the method is technically challenging, making reproducibility an issue (Beckmann and Soller, 1983).

Both isozymes and RFLPs are known as hybridisation-based systems. The final hybridisation-based system is minisatellite markers, which were discovered in the 1980s. Similar to RFLPS, minisatellites also require restriction enzyme digestion; however they look for polymorphisms in the number of tandem repeats of nucleotides rather than restriction fragment length. The main problem with this marker system was the non-random distribution of minisatellites across the whole genome.

In 1965, polymerase chain reactions (PCRs) enabled marker technology to become much more accessible. Prior to this, in order to have enough pure DNA for the three above marker systems to work required cloning or extracting large amounts of pure DNA. The main advantage of PCRs is that it has given researchers the ability to amplify any region of the genome for investigation. This also gave rise to PCR-based marker systems, such as microsatellite markers (otherwise known as simple sequence repeats – SSRs). Microsatellite markers are essentially minisatellite markers, except they can detect shorter sequences.

Random Amplified Polymorphic DNA markers (RAPDs) were the next PCR-based marker system to be discovered. RAPD amplifies total genomic DNA with a single random primer. Sequences amplified will be random and completely dependent on whether the primers complimentary sequence is present within the DNA being sampled. An advantage of this system was that prior knowledge of the organisms' DNA being analysed was not necessary. However, RAPDs are dominant markers meaning they are unable to differentiate between heterozygous and homozygous markers and there have also been considerable problems with their reproducibility (Jones *et al.*, 1997).

Amplified Fragment Length Polymorphisms (AFLPs) were introduced to increase the specificity of marker systems. AFLPs are essentially RFLPs that then undergo amplification via PCR. The increased specificity made this system highly reliable and reproducible, however it is a marker system that is again unsuitable for distinguishing between homozygous and heterozygous individuals (Collard *et al.*, 2005).

The third type of marker system that has been developed is the DNA-sequencing markers. These systems enable direct analysis of genetic variation in a much greater level of detail. This category includes single nucleotide polymorphisms, or SNPs. SNPs are able to detect polymorphisms directly in the DNA sequence at a single base (Jordan & Humphries, 1994). Thanks to their abundance and distribution within the genome, automation potential and low mutation rate they have rapidly become the most used type of genetic marker (Gupta, Roy and Prasad, 2001). Their amenability to automation has led to the development of many high-throughput genotyping methods, such as DNA chips, which has dramatically reduced the cost per data point for researchers whilst also increasing the speed of research being

undertaken (Boopathi, 2013). Kompetitive allele-specific PCR (KASP) markers (which are based on SNPs) and genotype-by-sequencing (GBS) are other high-throughput, DNA-sequence marker based systems that have dramatically increased options for using genetic markers in breeding and research alike (Semagn *et al.*, 2014).

Thanks to the improved sequencing of the wheat genome (IWGSC, 2018) and the advancement in molecular marker technology, it is now possible to generate ultra-high density linkage maps, detect and track the smallest introgressions in breeding programmes and construct more detailed and accurate genetic and physical maps of wheat. This research utilised SNP and KASP markers to detect and characterise introgressions from *Th. elongatum* 401007 in a wheat background (see section 6).

1.7.2. Genomic *in situ* hybridisation

The most common techniques utilised in research to detect chromosomal introgression or rearrangements in the wheat genome are C-banding, fluorescent *in situ* hybridisation (FISH) and genomic *in situ* hybridisation (GISH). All three of these techniques are known as cytogenetic techniques and involve staining the chromosomes of species for identification purposes.

Giemsa C-banding, commonly referred to as C-banding, was one of the first cytogenetic techniques used and worked by producing unique banding patterns on the heterochromatin of chromosomes. Whilst this approach is still useable today, it has mostly been replaced by the microscope based techniques GISH and FISH.

FISH can be used to identify the presence of wild relative DNA in wheat introgression lines. Fluorescently labelled probes bind to specific target sequences present on chromosomes, producing banding patterns unique to different species. If two species are very closely related these banding patterns become difficult to distinguish between. A more detailed summary of FISH is available in section 5.1.2.

GISH can also be used to identify introgressed segments from a wild relative of wheat in a wheat background by using fluorescently labelled total genomic DNA from the wild relative on metaphase spread preparations of the hybrid line. The probes bind to all corresponding chromosomes that match the probe DNA, rather than just specific target sequences in FISH. Whilst GISH is a highly valuable cytogenetic technique for visualising alien chromatin in a

wheat background, a more high-throughput methodology is required to increase the effectiveness of GISH in breeding programmes. A more detailed summary of GISH is available in section 5.1.3.

2. Aims and Objectives

The objectives of this research are as follows:

- To transfer chromosome segments spanning the entire genome of *Th. elongatum* (acc. 401007) into hexaploid wheat (cv. Paragon) using a whole genome introgression approach.
- To visualise and characterise introgressions using genomic *in situ* hybridisation.
- To use multi-coloured GISH to characterise the genome of the decaploid *Th. elongatum* (acc. 401007) and subsequent offspring lines with introgressions.
- To detect and characterise introgressions using the Axiom 35K SNP genotyping array.
- To develop KASP markers for future identification of homozygous and heterozygous introgressions in back-crossed generations.

3. General methods

The following research was initiated in October 2014 and concluded in August 2018 using experimental material from Glasshouses H1, H2 and H3 at Sutton Bonington Campus, University of Nottingham, UK. Germplasm generated was part of an active breeding programme; therefore the sowing and subsequent potting dates were staggered.

Methods outlined below are broad and relate to multiple investigations covered in this body of work. Specific methodology for each investigation is included in the relevant chapters.

3.1. Experimental Material

Genetic variation was introduced from decaploid *Th. elongatum* 401007 into a hexaploid wheat background by crossing *Th. elongatum* 401007 as the male parent with hexaploid wheat (acc. Chinese Spring) nullisomic for the *Ph1* locus. *Th. elongatum* 401007 was obtained from the USDA and the Chinese Spring wheat was obtained from the Germplasm Resource Unit (JIC). Subsequent offspring produced were known as F_1 hybrids and were put through several rounds of backcrossing with wild type hexaploid wheat (acc. Paragon) to reduce the size and number of segments from *Th. elongatum* 401007 in each line before finally allowing the plants to self-pollinate to produce germplasm with homozygous introgressions. A full crossing scheme is shown and explained in section 4.3.2.

3.2. DNA Extraction

Five young leaf segments, cut to approximately 2-3cm in length, were harvested into labelled 2ml tubes. Each tube had one 5mm stainless steel ball added and were freeze-dried overnight in the Christ Alpha 1-4 LD-Plus freeze-dryer (Martin Christ GmbH, Germany, Figure 3.1).

Samples were then ground to a fine powder by the Qiagen Tissue Lyser II at 25Hz/second for 6 minutes (Qiagen, Germany, Figure 3.2).



Figure 3.1: The Christ Alpha 1-4 LDplus Freeze Dryer.

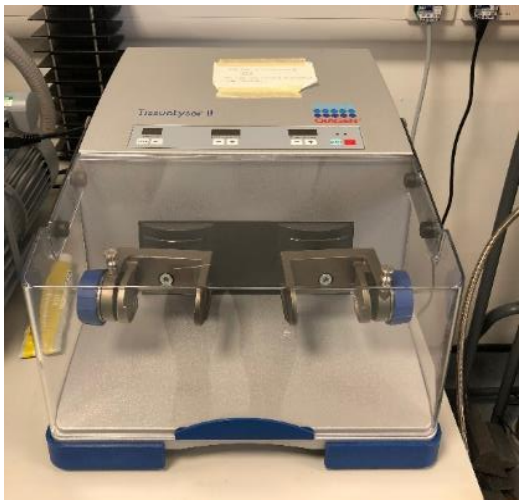


Figure 3.2: Qiagen TissueLyser II

600µl of extraction buffer (Appendix 9.1) was added; tubes were vortexed and then incubated for an hour at 65ºC. Samples were then put on ice for 15 minutes to lower to room temperature before adding 300µl of 6M ammonium acetate (Appendix 9.1). Samples were vortexed again and left for 30 minutes at 4ºC. Tubes were then centrifuged for 10 minutes at 13,000rpm before the supernatants were transferred to new 2ml tubes and 300µl of iso-propanol was added to each tube. The samples were thoroughly mixed and left so the DNA could precipitate for an hour at 4ºC.

Samples were centrifuged at 13,000rpm for 10 minutes and the supernatant tipped off. The pellets were washed twice in 300µl of 70% ethanol and then centrifuged at 13,000rpm for 3 minutes before tipping off the supernatant again. All ethanol was removed via pipette and the pellets were left to air dry at room temperature for 10 minutes. Once all ethanol had evaporated, each

pellet was re-suspended in 100µl de-ionised H_2O, vortexed briefly and left at 4ºC for an hour. Tubes were briefly vortexed and stored at -20ºC.

3.3. Quantifying DNA Concentrations

The concentrations of the purified DNA samples were measured using a Qubit 2.0 fluorometer (Invitrogen, California, Figure 3.3). Prior to measurement, the working solution for the Qubit was made in the ratio of 0.5 reagent dye: 200 buffer. 200µl of working solution was made for each sample and the two standards. For all measurements thin-wall PCR tubes (Invitrogen cat#Q32856 x500 tubes) were used. For the standards, 190µl of working solution was added to 10µl of the standards. For the samples, 199µl of working solution was added to 1µl of sample. All tubes were gently vortexed and centrifuged to remove any air bubbles prior to being measured. The Qubit was set up to carry out the DNA (ds) Broad Range assay, the two standards were measured first and then the samples followed. Once the DNA concentrations were determined the remaining DNA was stored at -20ºC. The protocol for the production of probes and blocking DNA produced from extracted DNA is explained in 5.3.4 and 5.3.5 respectively.



Figure 3.3: The Qubit 2.0 fluorometer

4. Germplasm Development

4.1. Introduction to the *Thinopyrum* genus

The genus *Thinopyrum* belongs to the *Triticeae* tribe and was segregated from the much larger genus *Elytrigia* in the late 20th century, alongside the genera *Lophopyrum* and *Trichopryum* (Baum & Johnson, 2017). The evolution and phylogenetic relationships of *Th. elongatum* with other *Triticeae* grass species is a highly debated issue and further molecular studies are required to resolve phylogenetic and functional relationships.

Apart from *Th. elongatum*, other noteworthy species within the *Thinopyrum* genus include: diploid *Thinopyrum bessarabicum* (Savul. & Rayss) Á.Löve (2n = 2x = 14, JJ), hexaploid *Thinopyrum intermedium* (Host) Barkworth & D.R.Dewey (2n = 6x = 42, $JJJ^{St}J^{St}StSt$) and decaploid *Thinopyrum ponticum* (Podp.) Liu & Wang (2n = 10x = 70, $JJJJJJJ^{S}J^{S}J^{S}J^{S}$).

The nomenclature and classification of these species has been, and still is, highly debated, dependent upon the method used to differentiate the species. Classification is prone to change when a new technology is discovered. To date there have been three 'phases' of classification: the first was morphological classification; the second phase, still implemented today, is cytogenetic classification using methods such as GISH and FISH; the third and most modern phase is the genomic phase (Baum & Johnson, 2017).

During the morphological classification stage, species from the genera *Agropyron, Lophopyrum, Thinopyrum, Elytrigia, Elymus* and *Trichopyrum* shared many characteristics and so were frequently referred to using multiple genera. For example, decaploid *Th. ponticum* is synonymous to *Agropyron elongatum* (Host) P.Beauv., *Elytrigia elongata* (Host) Nevisk., *Elytrigia pontica* (Podp.) Holub, and *Lophopyrum ponticum* (Podp.) Á Löve (He *et al.*, 2017). This can prove challenging when reading past literature, for example, diploid *Th. elongatum* can also be referred to as *Agropyron elongatum* and *Elytrigia elongata* (Linc, Sepsi and Molnár-Láng, 2012) .

The difficulties in classifying *Thinopyrum* species do not stop at nomenclature. Discerning genomic constitutions is equally challenging, especially with the polyploids. *Th. ponticum* is described with three diverging genomic constitutions, each supported by multiple studies: $E^bE^bE^bE^bE^bE^bE^bE^bE^bE^b$ (Muramatsu, 1990; Arterburn *et al.*, 2011),

$JJJJJJJ^sJ^sJ^sJ^s$ (Chen, Conner, Laroche, *et al.*, 1998), and $E^eE^eE^eE^eE^eE^eStStStSt$ (Zhang, Dong and Wang, 1996). Even clarity regarding the diploid species genomic constitution is debatable, with studies suggesting that the E and J genomes of *Th. elongatum* and *Th. bessarabicum* respectively are distinct enough to be classified in their own genera (Jauhar, 1990; Linc *et al.*, 2017); whilst other studies support the merging of the two into a single genus (Wang and Hsiao, 1989). An excellent summary of publications released on the investigations between the E and J genomes is available from Wang and Lu (2014). If the E and J genomes were re-designated as a common genome, then all polyploids within the genus would theoretically be autopolyploids. However, autopolyploids are usually limited in their genetic variation, a characteristic not true of the *Thinopyrum* polyploids (Jauhar, 1988).

4.1.1. The *Thinopyrum* species

This section introduces *Th. bessarabicum, Th intermedium, Th. ponticum and Th. elongatum* in more detail. These species are discussed as they either share genomes with *Th. elongatum* 401007 or are included in the Nottingham BBSRC wheat breeding programme. They also provide a more detailed overview of the *Thinopyrum* genus and the agronomic traits of interest held within.

Th. bessarabicum is a diploid, rhizomatous member of the *Thinopyrum* genus (2n = 2x = 14, JJ) with a perennial growth habit and well documented resistance to salinity (King *et al.*, 1997; Colmer, Flowers and Munns, 2006). *Th. bessarabicum* is also known to be resistant to various diseases, including FHB (Patokar *et al.*, 2016).

Th. intermedium, also known as intermediate wheatgrass, is a hardy perennial native to North America with a lifespan of up to 50 years (Jaikumar, Snapp and Sharkey, 2016). It has also been the subject of recent domestication attempts as a perennial food crop (DeHaan *et al.*, 2013). *Th. intermedium* can be described as a segmental allopolyploid member of the *Thinopyrum* genus (2n = 6x = 42, $JJJ^{st}J^{st}StSt$). *Th. intermedium* can be considered a segmental allopolyploid as; although it contains two distinct genomes (J and St); it also contains a modified genome (J^{st}) comprising of a J genome with a St centromeric region. It has been shown to possess drought and frost tolerance, increased photosynthetic and biomass productivity, and

numerous disease and pest resistances (see Table 4.1). A segmental allopolyploid shows some degree of homology between the genomes it consists of, meaning bivalents and multivalents can be formed.

The J genome within *Th. intermedium* has been shown to originate from the J genome of *Th. bessarabicum* and the St genome has been shown to originate from *Pseudoroegneria stipofolia* (M.Bieb) A.Love (Mahelka, Kopecký and Paštová, 2011). Recent studies have suggested that additional progenitors may have been involved in the evolution of *Th. intermedium*. GISH studies on *Th. intermedium* revealed the presence of *Dasypyrum villosum* (V genome), *Th. elongatum* (E genome) and *Ae. tauschii* (D genome) genomic material dispersed over 14 of *Th. intermedium*'s chromosomes (Mahelka, Kopecký and Paštová, 2011; Mahelka, Kopecký and Baum, 2013). High-molecular-weight glutenin subunit studies have also supported the involvement of *Ae. tauschii* in the evolution of *Th. intermedium* (Cao *et al.*, 2014). Therefore, the genomic constitution of *Th.intermedium* may not be as resolved as previously thought.

Th. ponticum is a decaploid species within the *Thinopyrum* genus that, asides for wide hybridisation with wheat, is currently used for soil erosion control, forage grass, a source of cellulosic ethanol (biofuel) and to produce particle board. It is currently one of the most saline tolerant of commercially available plants (6 cultivars have been released in the US & Canada) and can be grown on saline, sodic or semi-arid soils (USDA, 2014). Accessions range in height from 3-10 feet tall and display a perennial nature alongside extensive disease resistance traits, yield improvement traits and abiotic stress tolerance (Li and Wang, 2009; Ceoloni *et al.*, 2014). As mentioned previously, there have been three diverging genomic constitutions described in studies, $E^bE^bE^bE^bE^bE^bE^bE^bE^bE^b$ (Arterburn *et al.*, 2011), $JJJJJJJ^sJ^sJ^sJ^s$ (Chen, Conner, Laroche, *et al.*, 1998), and $E^eE^eE^eE^eE^eE^eE^e$StStStSt (Zhang, Dong and Wang, 1996). Based on studies using a variety of molecular technology, cytogenetic studies and phylogenetic studies the first genomic constitution ($E^bE^bE^bE^bE^bE^bE^bE^bE^bE^b$) can be considered disproved, *Th. ponticum* is not an autodecaploid (Zhang, Dong and Wang, 1996; Brasileiro-vidal *et al.*, 2003). Remaining options are still being investigated (He *et al.*, 2017).

Th. elongatum is a wild relative of wheat that is native to Eurasia. Literature reports that the species contains diploid, tetraploid, hexaploid and decaploid accessions (Mao *et al.*, 2010; Chen *et al.*, 2013; Guo *et al.*, 2016). More

commonly known as tall or bunched wheatgrass, accessions range in height from 1-3 metres and have pale, ribbed leaves approximately 5mm wide. *Th. elongatum* is a perennial species with spiked inflorescence up to 30cm long, with alternating spikelets up to 3cm long (Figure 4.1 and Figure 4.2). Each spikelet is hermaphroditic, capable of outbreeding and inbreeding, with tightly adhered glumes, making threshing difficult (Marais *et al.*, 2014). Most research reporting on *Th. elongatum* has been carried out on the diploid (2x = 2n = 14, EE), including the description of the E genomes first ever karyotype (Linc, Sepsi and Molnár-Láng, 2012). The accessions used within this research include the decaploid *Th. elongatum* 401007 and the diploid *Th. elongatum* 571718. Seed was obtained from the Genebank, USDA-ARS Beltsville Md., and USA. At the USDA seedbank there are currently 54 accessions of *Th. elongatum,* 374 accessions of *Th. intermedium*, and 9 accessions of *Th. ponticum*.



Figure 4.1: Photo of multiple *Th. elongatum* fertile heads grown in the glasshouses at University of Nottingham.



Figure 4.2: Macro photo of a fertile *Th. elongatum 401007* head showcasing the species protruding anthers at anthesis.

4.1.2. Using *Thinopyrum* species to enhance wheat diversity

Despite the classification issues, what the majority of research does agree on is that species from within the *Thinopyrum* genus offer a large amount of variation in virtually all agronomic traits of interest to utilise in improving, or protecting, elite wheat cultivars (see Table 4.1). In addition to the reservoir of beneficial agronomic traits within the *Thinopyrum* genus, the J and E genomes within the genera have been shown to be highly related to the three wheat genomes A, B and D (Liu, Li and Zhang, 2007). This suggests species within the Thinopyrum genus are more suitable for wide hybridisation with wheat than other wild relatives with more distinct genomes (e.g. *S. cereale,* 2n = 2x = 14, RR). Most studies carried out on the phylogenetic relationships between *Thinopyrum* and *Triticum* species have focussed on the diploids, with very little data investigating the phylogenetic relationships of the polyploid Thinopyrum species (Guo *et al.*, 2016). However, most of the studies done to transfer traits of interest from *Thinopyrum* species have focussed on those with higher ploidy such as *Th. ponticum* and *Th. intermedium*. Due to the potential identification issues of the accession *Th. elongatum* 401007, previous research on *Th. elongatum, Th. intermedium* and *Th. ponticum* is presented here to highlight the importance of the whole genus as a source for improving genetic diversity in the wheat gene pool regardless of classification difficulties.

Table 4.1: A brief summary of agronomic traits investigated and confirmed in the *Thinopyrum* species. The gene and chromosome transferred is included if published.

Species	Trait transferred	Gene transferred	Chromosome transferred	Reference
Th. elongatum	Perennial growth habit	-	4E	(Lammer *et al.*, 2004)
	Salt tolerance	-	3E	(Colmer, Flowers and Munns, 2006)
	FHB Resistance	-	7ES	(Fu *et al.*, 2012)
Th. intermedium	FHB Resistance	-	-	(Turner *et al.*, 2013)
	Leaf rust resistance	*Lr38*	7Ai#2	(Friebe *et al.*, 1992)

	Stem Rust resistance	*Sr44*	7J#1S	(USDA, NIFA and BGRI, 2018)
	BYDV Resistance	*Bdv2*	7Ae#1	(Banks *et al.*, 1995)
	Stripe Rust Resistance	*Yr50*	-	(Liu *et al.*, 2013)
	WSMV Resistance	*Wsm1*	4S	Liang *et al* (1979)
	High Grain Protein	-	-	(Cao *et al.*, 2014)
	Powdery Mildew Resistance	*Pm40*		(Luo *et al.*, 2009)
		Pm43		(He *et al.*, 2009)
Th. ponticum	Leaf rust resistance	*Lr19*	7Ae#1	(Sharma and Knott, 1966)
		Lr24	3Ae#1	(Sears, 1977)
		Lr29		
	Stem rust resistance	*Sr24*	3Ae#1	(Jiang, Friebe and Gill, 1994)
		Sr25	7Ae#1L	(McIntosh, Dyck and Green, 1977)
		Sr26	6Ae#1L	(Friebe *et al.*, 1994)
		Sr43	7el#2	(Niu *et al.*, 2014)
	Eye spot resistance	-	4Ai#2	(Li *et al.*, 2004)
	Cephalosporium stripe resistance	-	6Ae#2	(Cai, Jones and Murray, 1996, 1998)
	FHB Resistance	-	-	(Oliver *et al.*, 2006)
	Semi-dwarfing phenotype	-	-	(Chen *et al.*, 2012)
	Salt tolerance	-	-	(Guo *et al.*, 2015)
	Powdery Mildew Resistance	*Pm51*	-	(Zhan *et al.*, 2014)

4.2. Aims and Objectives

The aims and objectives of this chapter are:

- Introduce and explain the breeding programme used to generate wheat (cv. Paragon)/ *Th. elongatum* 401007 introgression lines.
- Analyse the success of the breeding programme in terms of number of lines and seed produced.
- Present fertility rates of different generations throughout the backcrossing programme.

4.3. Materials and Methods

The Nottingham BBSRC Wheat Research Centre (WRC) is currently developing genome-wide introgression lines produced via a whole genome shotgun approach between wheat and ten wild relatives of wheat. The wild relatives included in the programme are: *Am. muticum, Ae. speltoides, Ae. caudata, T. timopheevii, T. urartu, S. cereale, Th. bessarabicum, Th. elongatum, Th. intermedium* and *Th. ponticum* (King *et al.*, 2017). This chapter will focus on the production of wheat/*Th. elongatum* 401007 hybrids.

4.3.1. Initial germplasm

Two varieties of spring wheat were used during this research: *ph1* mutant Chinese Spring (Collection id: WPGS ID#28124) was used to create the initial F_1 hybrids and then wild-type Paragon (Collection id: BBSRC_Wheat (ID#23201)) was used for subsequent back-crosses at all stages of the breeding programme. Both cultivars of wheat were obtained from the Germplasm Resource Unit (JIC). Only one accession of *Th. elongatum* was used for the breeding programme, *Th. elongatum* 401007. Additional wild relative species and accessions were used in the multi-colour GISH characterisation, all provided by the USDA seedbank. All species and accessions used are summarised in Table 4.2.

Table 4.2: All species and accessions of the wild relatives used in this research, including the country of origin, the ploidy and chromosome number and genomes known to be in that species.

Species	Accession number	Ploidy	Chromosome number	Genomes Known
Thinopyrum elongatum	401007	Decaploid (with aneuploidy)	72	E, St
Thinopyrum elongatum	531718	Diploid	14	E
Thinopyrum bessarabicum	531711	Diploid	14	J
Pseudoroegneria stipofolia	531058	Diploid	14	St
Thinopyrum ponticum	531717	Decaploid	70	J, St

Figure 4.3 shows the adult phenotypes of the diploid and decaploid *Th. elongatum* accession used in this breeding programme, along with *Th. ponticum* 531737 for a phenotypic comparison, *Ps. stipofolia* 531058 - a suspected progenitor of the decaploid species, and the wheat cultivar Paragon.

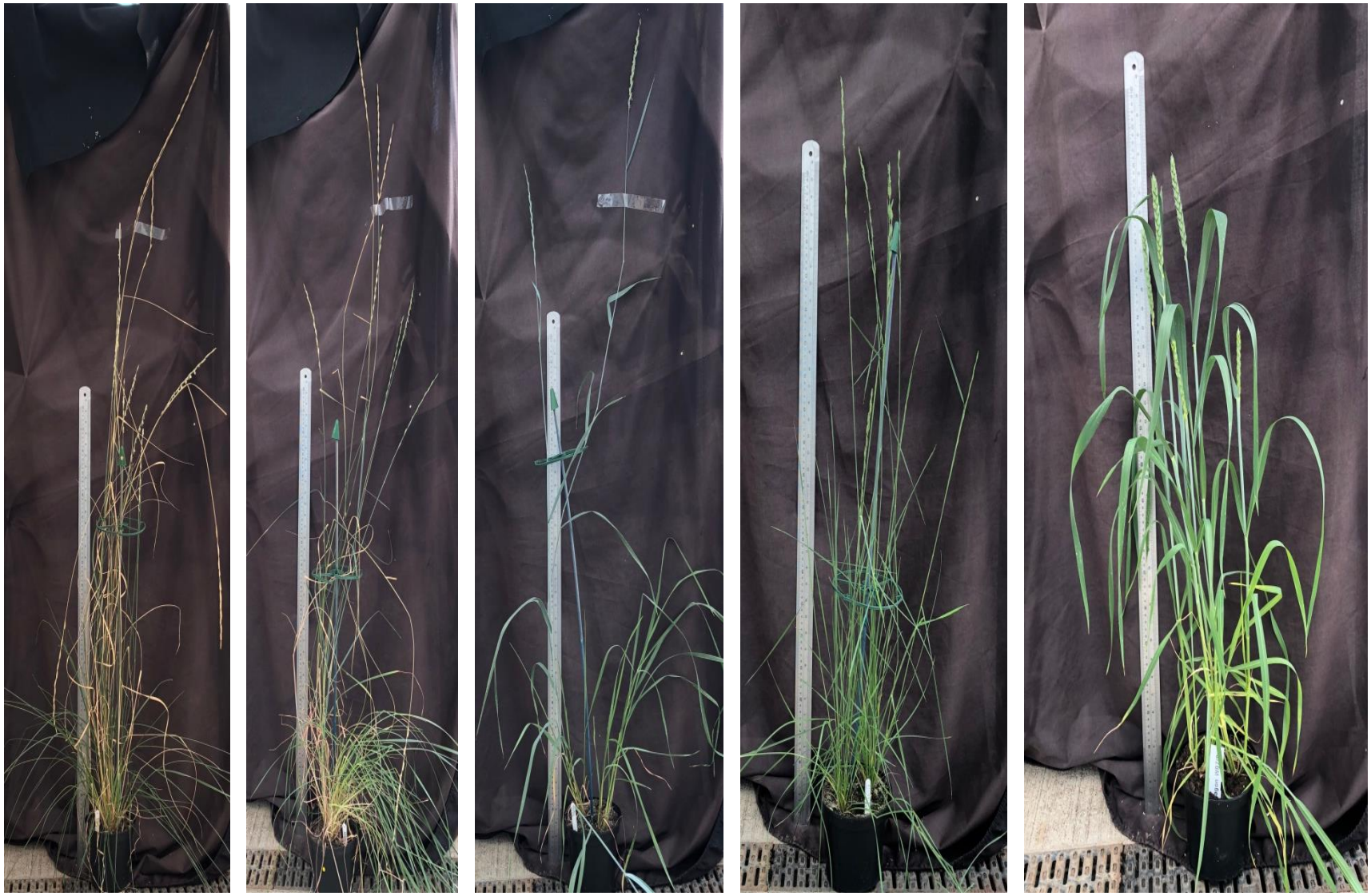


Figure 4.3: From left to right, *Th. elongatum* 401007, *Th. ponticum* 531737, *Ps. stipofolia* 531058, *Th. elongatum* 531718 and *T. aestivum* (acc. Paragon W07).

4.3.2. Crossing Scheme

All the germplasm was generated from an initial cross between wheat (var. Chinese Spring), homozygous for the *ph1/ph1* mutation, and *Th. elongatum* 401007. The *ph1/ph1* wheat was pollinated by the wild relative to produce inter-specific F_1 hybrids. The F_1 hybrids produced were essentially haploid, with one complete genome from each parent (see Figure 4.4).

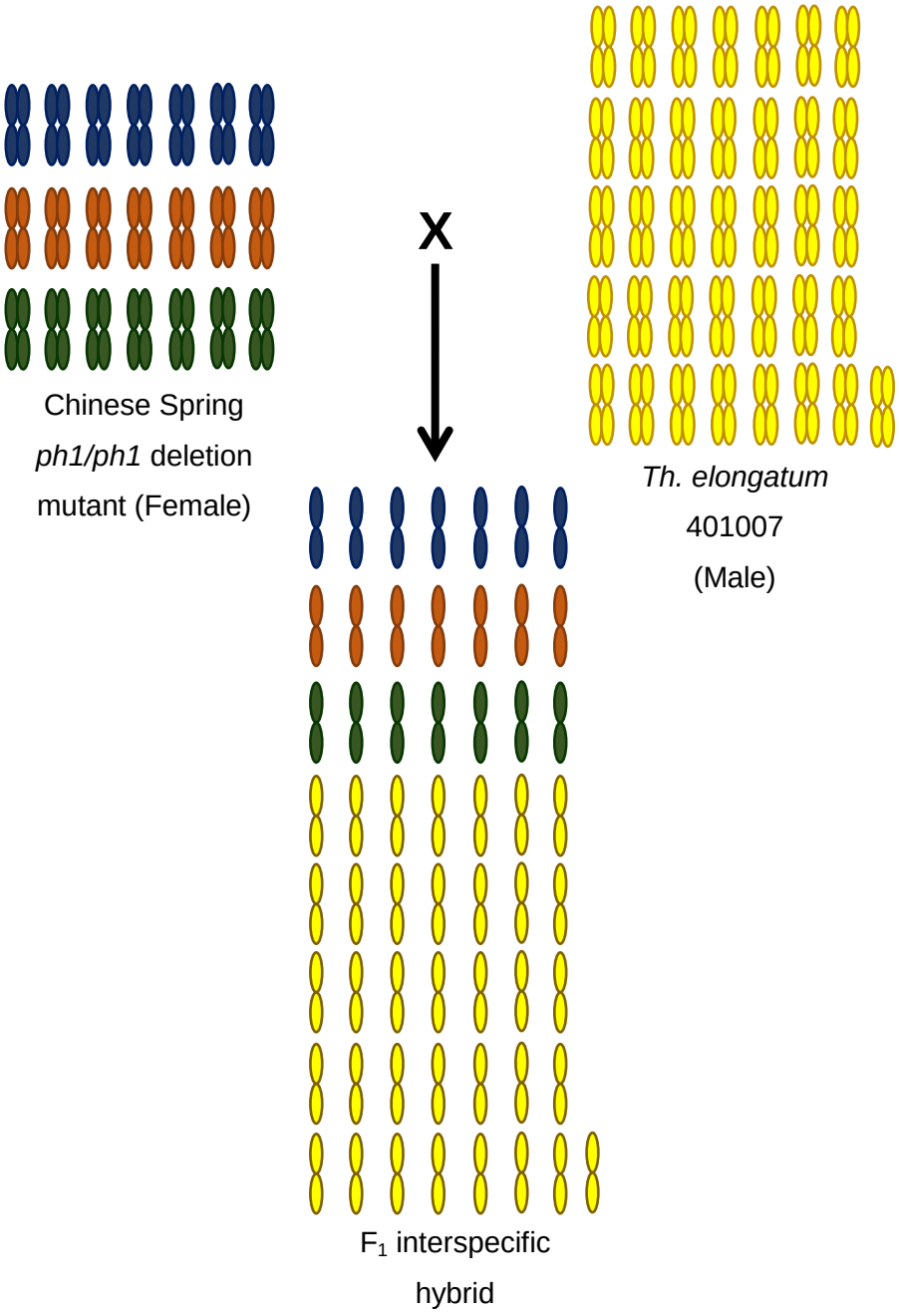


Figure 4.4: Diagrammatic representation of the initial cross to produce an interspecific F_1 hybrid between *ph1/ph1* wheat and *Th. elongatum* 401007. The blue, green and orange chromosomes represent the A,B and D genomes of wheat respectively. The yellow chromosomes represent those from *Th. elongatum* 401007.

To generate successive progeny, the interspecific F_1 hybrids were crossed to wild-type wheat (cv. Paragon) to generate back-crossed lines (BC_1). These BC_1 lines often had large and numerous introgressions present in a wheat background, and so subsequent rounds of back-crossing with wheat (cv. Paragon) was undertaken to reduce the number and size of these segments. The crossing programme is summarised in Figure 4.5.

The use of the *ph1/ph1* deletion mutant for the initial cross should enable the homoeologous recombination of the *Th. elongatum* chromosomes with wheat, enabling the potential transfer of the entire *Th. elongatum* genome via a 'shotgun' approach.

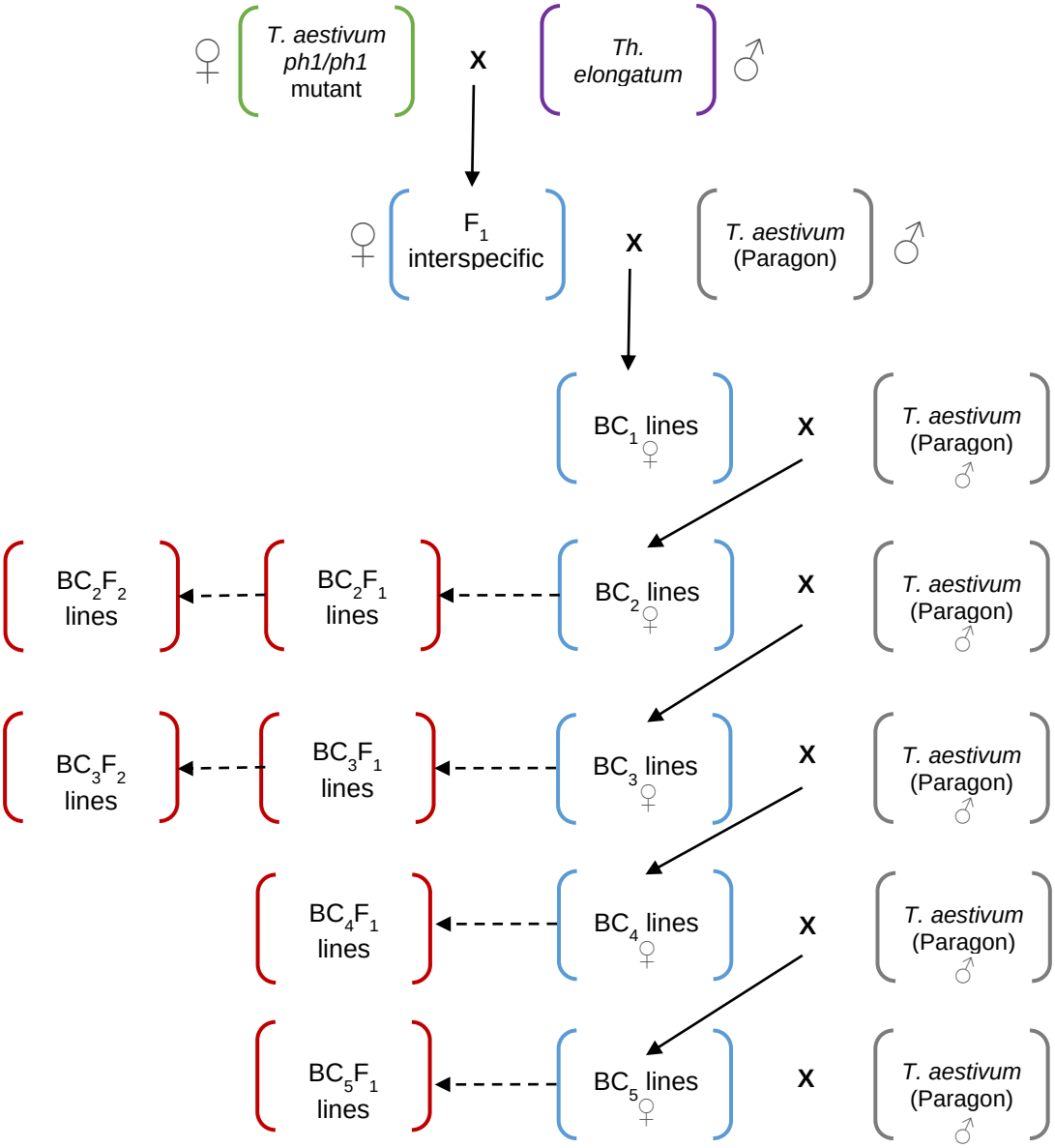


Figure 4.5: An overview of each stage of the breeding programme for *Th. elongatum* 401007. Solid arrows represent production of the next generation via crossing. Dashed arrows represent the production of subsequent generations via self-fertilisation. The variety of wheat used at each stage is shown and the gender role the plants play at each stage is also shown.

4.3.3. Germination

Seeds were germinated on filter paper in Petri dishes. To initiate germination the filter paper was wet thoroughly with de-ionised water, seedlings were placed in the correctly labelled Petri dishes and then kept at 4ºC for 2-3 days before being moved to a 20ºC dark growth room. Radicle and coleoptile emergence would typically occur within a week.

4.3.4. Vernalisation and Plant Growth Conditions

Once the seedlings reached approximately 2-3 inches in height, the seedling trays were transferred to vernalisation chambers. The entry dates were recorded and trays were kept in vernalisation for 6-8 weeks dependent on the generation of the backcross. The vernalisation chamber maintained conditions between 6-8ºC with a photoperiod of 12 hours.

Once vernalisation requirements were met, seedlings were transferred to individually labelled 2 litre pots in the glasshouse with John Innes No.2 compost. Hortifeed nutrient feed was supplied by a drip system to the plants throughout their growth cycle in the glasshouse. Temperatures were maintained at 21ºC and white lights were used to supplement shorter day lengths to deliver a 16 hour photoperiod (monitored by the automated glasshouse system).

4.3.5. Production of F_1 hybrids

To produce the F_1 interspecific hybrids pollen was taken from *Th. elongatum* 401007 ears and was used to pollinate emasculated spikes of *ph1* mutant Chinese Spring. Any seed produced would grow to form an F_1 hybrid plant.

4.3.5.1. Emasculation

Using forceps any florets in addition to the primary and secondary florets were excised. The remaining primary and secondary florets were then emasculated prior to anthesis by using the forceps to remove the immature anthers without damaging the developing stigma. To prevent unplanned pollination by any plants within the same glasshouse, emasculated ears were covered with glassine bags.

4.3.5.2. Pollination

Once stigmas reached maturity (became 'fluffy', see Figure 4.6), florets were pollinated with *Th. elongatum* pollen, given an individual label to identify the cross and re-covered with a glassine bag (Figure 4.7).



Figure 4.6: Macro image of a self-pollinating back-crossed line. The white fluffy stigmas are clearly visible protruding from within the florets, signifying fertility.



Figure 4.7: An example of a glassine bag covered head from a back-crossed line, with a unique identifier label after pollination.

4.3.6. Production of subsequent lines

All subsequent back-crossed generations (F_1, BC_1, BC_2 etc) were emasculated and pollinated as described above, with the exception that they were backcrossed as the female and pollen used was from wheat (var. Paragon). Figure 4.8 shows the ears of BC_1, BC_2 and BC_3 wheat/*Th. elongatum* lines, showing the progression in morphology towards something more recognisably wheat.

Self-fertilised generations (e.g. BC_2F_1, BC_2F_2) were produced by bagging ears prior to anthesis without emasculating and allowing them to self-pollinate.



Figure 4.8: Heads from a BC_1, BC_2 and BC_3 *T. aestivum/Th. elongatum* line. All heads shown were post anthesis and left to self-fertilise if capable. The morphology clear becomes more 'wheat-like' from left to right.

4.3.7. Threshing

Once the entire plant was ready to harvest, individually labelled heads inside their glassine bags were removed from their tillers and placed into corresponding labelled bags. Each ear was then threshed by hand, by removing all the florets from the spikelet and removing the seeds from each floret. The threshed seeds were placed into a labelled brown paper envelope and stored in the seed store until required. The seed store was kept at ~3°C with a relative humidity of 5%.

4.3.8. Observations

At each generation, the number of crosses or self-fertilised ears was recorded, along with the number of seed obtained back from each cross or self. This gave an indicator of fertility and was calculated as:

$$\text{Crossed Ear Fertility} = \frac{\text{number of ears producing viable seed}}{\text{total number of crossed ears}}$$

$$\text{Self-fertilised Ear Fertility} = \frac{\text{number of self-fertilised ears producing viable seed}}{\text{Total number of self-fertilised ears}}$$

The average number of (crossed) seeds produced at each generation was calculated as:

$$\text{Average Seed Set} = \frac{\text{Total number of seed produced in one generation}}{\text{Number of crosses done in one generation}}$$

4.4. Results

To date 1,883 crosses have been completed giving 8,425 seed, and 1,796 self-fertilised heads have given 55,683 seed. All seeds were produced from the single *Th. elongatum* accession 401007. In total, 27 crosses were made between *Th. elongatum* 401007 and *ph1* deletion mutant wheat (cv. Chinese Spring) producing 222 F_1 seed. From these 222 seed, 75 were randomly selected and sown, with 43 reaching maturity. From these 43 plants, 1,002 BC_1 seed were produced from 182 crosses. 88 BC_1 seed were sown after random selection, of which 80 germinated. From 633 crosses on the BC_1, 1,604 BC_2 seed were produced and from these seeds 158 were randomly selected and sown. 130 BC_2 seedlings reached maturity and produced 3,789 BC_3 seed from 709 crosses. For the BC_3 generation, 227 randomly selected seeds were sown, of which 153 reached maturity and set seed, producing 870 BC_4 seed from 208 crosses. From the 870 BC_4 seed, 128 were sown and 108 survived to maturity and produced 938 seed from 128 crosses. 112 BC_5 seed were sown, of which 90 survived to maturity. No crosses were carried out on the BC_5 seedlings and so the only seed set was from self-fertilised heads.

The number of seed sown, germinated and heads producing seed from both crosses and self-fertilised florets for each stage of the breeding programme is summarised in Table 4.3.

Table 4.3: Seed data for each generation of the introgression breeding programme between *Th. elongatum* 401007 and *T. aestivum*. Numbers followed by * represent a minimum for that value due to unrecorded data.

	No. seed sown	No. germinated (%)	No. of crosses producing seed (%)	Average seed set per cross (No. of seed/ total number of crosses)	No. of 'selfs' producing seed (%)	Average seed set per self (No. of seed/ total number of 'selfs')
F_1	75	43 (57.3)	157/178 (88.2)	5.6 (1002/178)	67/87 (77.0)	4.6 (399/87)
BC_1	88	80 (90.9)	356/633 (56.2)	2.5 (1604/633)	86/118 (72.9)	7.5 (890/118)
BC_2	158	130 (82.3)	544/709 (76.7)	5.3 (3789/709)	131/156 (84.0)	6.1 (953/156)
BC_2F_1	315	240 (76.2)	n/a	n/a	313/361* (86.7)	61.2 (22,261*/361*)
BC_2F_2	680	519 (76.3)	n/a	n/a	620/684* (90.6)	28.4 (19,411/684)
BC_3	227	153 (67.4)	138/208 (66.3)	4.2 (870/208)	108/117* (92.3)	20.2 (2,363/117)
BC_3F_1	71	56 (78.9)	n/a	n/a	55/57* (96.5)	35.0 (1997/57)
BC_4	128	108 (84.4)	115/132 (87.1)	7.1 (938/132)	134/144* (93.1)	32.2 (4641/144)
BC_4F_1	109	99 (90.9)	n/a	n/a	36/36* (100)	36.6 (1317/36)
BC_5	112	90 (80.4)	n/a	n/a	34/34* (100)	40.4 (1374/34)
BC_5F_1	15	12 (80)	n/a	n/a	2/2* (100)	38.5 (77/2)

The lowest germination rate came from the F_1 generation (57.3%). The subsequent generations had the germination rates of 90.9% (BC_1), 82.3% (BC_2), 76.2% (BC_2F_1), 76.3% (BC_2F_2), 67.4% (BC_3), 78.9% (BC_3F_1), 84.4% (BC_4), 90.9% (BC_4F_1), 80.4% (BC_5) and 80% (BC_5F_1) (Figure 4.9). Overall, no trend in germination rate was observed.

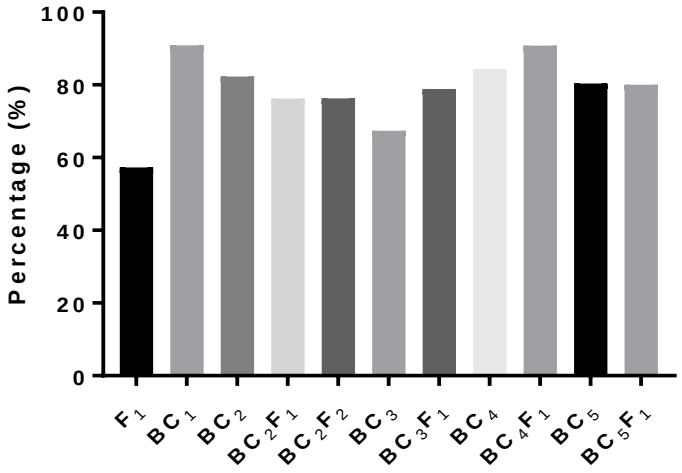


Figure 4.9: Germination rates as a percentage for each generation in the breeding cycle.

Figure 4.10 shows the ear fertilities from (a) crossed heads and (b) self-fertilised heads expressed as a percentage. Overall, results show no increase in crossed head fertility as the breeding programme progresses due to a spike at the F_1 generation and a dip at the BC_3 generation. The figure does show a strong positive trend in self-fertilised ear fertility suggesting it increases as plants progress through the breeding programme.

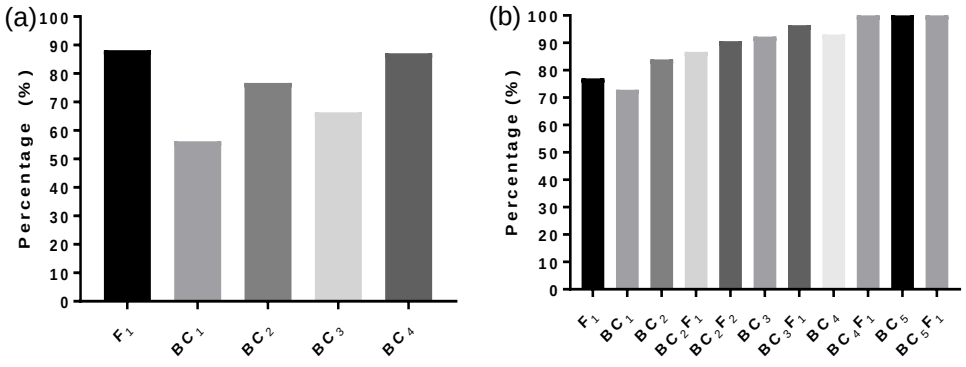


Figure 4.10: The ear fertility of (a) crossed heads and (b) self-fertilised heads represented by the percentage that successfully resulted in seed production at each stage of the breeding programme.

Figure 4.11 shows the average number of seed produced from (a) crossed heads and (b) self-fertilised heads. Both graphs show a general increase in seed production, except for the dip in crossed seed produced by the BC_3 population and the BC_2F_1 population showing a spike in self-fertilised seed set.

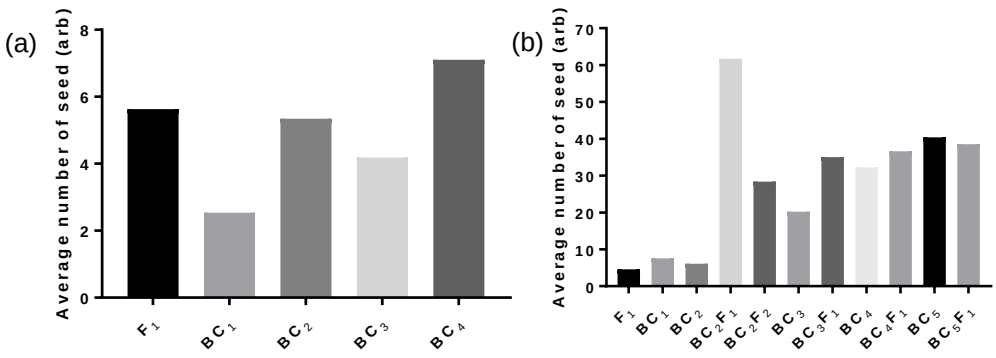


Figure 4.11: Average number of seed produced from (a) crossed heads and (b) self-fertilised heads at each successive generation.

4.5. Discussion

The breeding programme undertaken in this research used a shotgun introgression approach to produce lines of wheat with introgressions from *Th. elongatum,* which ideally span the entire *Th. elongatum* genome. The *ph1/ph1* wheat used to create the initial F_1 hybrids enables the formation of F_1 gametes capable of homoeologous recombination. This means that the entire *Th. elongatum* genome can be transferred into a wheat background. Previous breeding programmes utilising wild relatives of wheat have had some success at introgressing traits into a wheat background (see Table 4.1). However, usually the approaches taken have focused on transferring a specific trait of interest, such as rust resistance, and therefore are not utilising the whole range of genetic variation available in the wild relative genome. By producing introgressions in a wheat background from across the entire *Th. elongatum* genome, progeny produced can be analysed in the field for various phenotypic traits, including disease resistances and abiotic stress tolerances, and therefore the entire genome of *Th. elongatum* can be analysed for advantageous agronomic traits of interest. This will not be a simple task, as some lines produced with multiple segments from wild relatives in a wheat background may display complex changes in phenotype from interactions of multiple genes. Therefore producing lines with single introgressions would be the most beneficial for phenotypic characterisation. The production of introgressions in overlapping segments using this breeding strategy would allow the production of smaller introgressions by crossing the two overlapping lines, thereby reducing the impact of linkage drag and allowing any changes in phenotype to be accredited to a more specific section of *Th. elongatum* chromatin.

Th. elongatum 401007 was the single accession used in this research for the production of the back-crossed generations. This was due to it being one of the first accessions obtained from the USDA seed bank and the successful production of F_1 interspecific hybrids. It was initially thought to be a diploid accession, however metaphase root spreads and chromosome counts quickly revealed it to be a decaploid with aneuploidy, giving it 72 chromosomes in total. Using a decaploid accession with debatable genomic constitution has made subsequent cytogenetic work and genotyping work on the introgressions produced more challenging. However, using a decaploid

accession has also resulted in the production of over 1,500 individual plants with a surprisingly high number of introgressions.

The wheat accession chosen for creating the initial F_1 interspecific hybrid was *ph1* mutant Chinese Spring. Chinese Spring was chosen because the *ph1/ph1* deletion mutant was already available and had already been used in genetic studies. It was also the same accession investigated by Sears (1976) whilst studying the genetic control of chromosome pairing in wheat and, for the past 20 years, Chinese Spring has also been the reference hexaploid wheat line for genetic studies (Uauy, 2017). The lack of the *Ph1* locus and any homologous chromosomes enabled homoeologous recombination to occur between the *Th. elongatum* and *T. aestivum* chromosomes when crossed within the F_1 gametes. Subsequent back-crosses were carried out using wild type *T. aestivum* (acc. Paragon). Paragon was chosen for several reasons including: improved agronomic performance for traits such as standing power in comparison to Chinese Spring (Willow, 2013), being a previous elite UK spring wheat cultivar, and its suitability to wide hybridisation and subsequent trait analysis (Moore, 2015). It too has now had its genome sequenced and a reference assembly is available for analysis (Uauy, 2017).

Due to the absence of homologous chromosomes during the initial generation of F_1 hybrids, homoeologous recombination was forced during gamete production. This approach was predicted to result in an increased number of introgressions in comparison to other methods such as radiation treatment. The resulting progeny of such crosses were expected to struggle with genome stability and fertility issues, as mismatched chromosomes could be less likely to successfully complete meiosis, resulting in unviable gamete production. However, the F_1 interspecific hybrids between *T. aestivum* and *Th. elongatum* showed a surprisingly high germination rate of 57.3%, in comparison to the F_1 germination rate for *Amblopyrum muticum* interspecific hybrids of 28.6% (King *et al.*, 2017). In addition to a higher than expected germination rate, the F_1 interspecific hybrids also had a much higher crossed ear fertility than expected at 88.2%. This differed greatly from other wild relative species within the breeding programme: *Am. muticum* had a crossed ear fertility of just 16.2% (King *et al.*, 2017) and *Th. bessarabicum* F_1 fertility rates were only 1.6% (Grewal *et al.*, 2018). This increase in fertility at such an early state of the breeding programme could possibly be attributed to the close synteny between the E and St genomes of *Th. elongatum* 401007 and

the D genome of *T. aestivum* (Wang, 2011). If during chromosomal pairing at meiosis I, the D genome is capable of forming bivalents with the E and St genomes present in *Th. elongatum* 401007, meiosis could be able to complete as normal, thus explaining the maintained fertility of the interspecific hybrids. This could also explain the increased number of introgressions present in *Th. elongatum* hybrid lines in comparison to other wild relatives, such as *Secale cereale* (Heath, 2018). However, at this point unpaired A, B and D chromosomes would still need to be resolved during meiosis and translocations could be present within these chromosomes – any unresolved pairing or univalents could result in unstable meiosis and unviable gametes. It could simply be down to luck that the chromosomes assembled at the same end of the spindle fibre to form viable gametes during meiosis I in the F_1 gametes.

In total, 10 wild relative species including *Th. elongatum* and *Am. muticum* were used in the introgression breeding programme at the Nottingham/BBSRC Wheat Research Centre. The F_1 interspecific hybrids produced from *Th. elongatum* were the only ones to show male fertility. This enabled some ears to be self-fertilised, and for the crossing to be done in reverse, meaning the F_1 interspecific hybrid could be used as the male pollen donor as well as the female receptor. Future work could be to investigate the effect of using *Th. elongatum* F_1 hybrids as male pollen donors on the number of introgressions present in the back-crossed lines to see if this increases or decreases the number of introgressions present in offspring. This work was not part of this project as the BC_1 plants used in this crossing programme were those produced using the F_1 plants as the female parent.

The general trend in increasing ear fertilities and seed set suggest an increased genetic stability and increase in overall plant vigour as the breeding programme progresses. Lines through the breeding programme showed a variation in chromosome number, however as the number of cycles completed in the crossing programme increased, the chromosome number generally decreased towards 42. Therefore, the improvement in plant vigour could be partly explained by the gradual restoration of the back-crossed lines to the normal wheat chromosome number of 42 with each round of crossing.

The BC_3 generation did not perform as well as expected, with reduced levels of germination, ear fertility and seed set. The reduction in ear fertility and seed set can be partly attributed to a Hessian Fly infestation that infected the

glasshouse during the early growth stages of the BC_3 population; thus causing a significant number of plants to struggle to reach sexual maturity (see Figure 4.10 and Figure 4.11). The reduction in BC_3 germination rate cannot be attributed to the Hessian Fly infestation, but could in part be attributed to sub-optimal germination conditions or seedlings with reduced vigour being randomly selected from the BC_2 offspring.

Similarly, the spike in seed production in the BC_2F_1 population could suggest that this generation was particularly productive, but it could also be attributed in part to unrecorded data and lack of disease in the glasshouse during their lifecycle.

The shotgun introgression approach used in the breeding programme was successful in generating a large number of back-crossed lines with *Th. elongatum* genomic material in a *T. aestivum* background. Lines produced with multiple introgressions and/or whole chromosome additions are not ideally suited for commercial exploitation and make subsequent cytological and genotyping methodologies to determine beneficial traits difficult to achieve. Ideally, future work will involve continuing the breeding of lines with multiple segments from *Th. elongatum* to single segments, which are then made homozygous by self-fertilisation in order to stabilise the genetics of the line. Introgressions produced in this research have been visualised using cytogenetic techniques such as genomic *in situ* hybridisation (see section 5) and molecular markers are under development to enable high throughput screening for rapid detection of future introgressions (see section 6).

5. Visualising Introgressions using Cytogenetics

5.1. An Introduction to Cytogenetic Techniques

The field of cytogenetics originated in the early 20th century soon after the discovery that genes were located within chromosomes. The combination of cytology and genetics produced the hybrid subject area cytogenetics, and researchers became focussed upon chromosome identification and characterisation. This chapter will briefly look at the history and previous uses of cytogenetics in wheat research. Prior to the 1970's, chromosome analysis was limited to classical cytogenetic methods, including comparisons of chromosome size, location of constrictions and pairing studies (Silva and Souza, 2013). The development of molecular cytogenetics allowed the identification of specific sequences by directly staining the chromosomes.

5.1.1. Giemsa C-banding

Giemsa C-banding is a molecular cytogenetic technique, more commonly known as C-banding. It is a selective chromosome staining technique that stains heterochromatin, producing visible 'bands' across a chromosome. The technique enabled researchers, not only to stain chromosomes, but to distinguish between individual chromosomes. It was first developed and utilised in 1970 to label mouse satellite DNA on chromosomes *in situ* (Pardue and Gall, 1970). The constitutive heterochromatin banding patterns differ between individual plant species and so differences can be observed and utilised for identification purposes. Variation in banding patterns also enabled translocations, additions, inversions or deletions to be identified. However, C-banding is very technically challenging and the resolution of C-banding is limited, as the number of bands produced on chromosomes could be very low, which makes detecting any chromosomal rearrangements difficult. The detection of banding patterns was initially done via radiation, making the method more hazardous than more advanced cytogenetic techniques that use fluorescent labels rather than radioactive labels.

Whilst now being surpassed by more advanced techniques, C-banding can still be effective when used in sequential analysis with GISH or FISH to identify alien addition and substitution lines. For example, sequential C-banding and GISH was used to identify substitution lines of *L. ponticum* (Cai *et al* 1996).

5.1.2. Fluorescent *in situ* hybridisation

Fluorescent *in situ* hybridisation (FISH) is a molecular cytogenetic technique that uses fluorescent probes to bind to specific sequences along the length of the chromosome producing a unique banding pattern. FISH was first developed in the area of human molecular cytogenetics in 1986 (Pinkel, Straume and Gray, 1986). Progression to using this methodology in plant molecular studies was held up from difficulties obtaining suitable samples without cell wall or cellular debris. The use of radioisotopes in C-banding was much more time-consuming than the fluorescent-labelled probes used in FISH, making FISH a more efficient methodology. FISH also had a higher resolution than that of C-banding, as it could be used to detect specific nucleic acid sequences.

The fluorescent-labelled probes used in FISH could be made from species-specific repetitive sequences, ribosomal genes or unique sequences. By using FISH probes designed from repetitive sequences dispersed throughout the chromosomes, the banding patterns produced could be used to identify individual chromosomes (e.g. 1E is distinguishable from 2E due to different banding patterns produced). It can also reveal intergenomic chromosome rearrangements, but this can be limited depending on how distinguishable the FISH patterns are. By using species-specific repetitive sequences, FISH probes can also be used to identify wild relative addition, translocation and introgression lines. Another useful application of FISH is using ribosomal sequences for karyotype analysis and subsequent comparative phylogenetic analysis.

For the *Thinopyrum* genus, establishing phylogenetic relationships and genomic constitutions has been a challenge for taxonomists and geneticists as discussed in detail in section 4.1. To help resolve these problems, recent research has been focussed on discovering karyotypes of diploid progenitors of the genus. To date, a karyotype of the E genome (see Figure 5.1) from diploid *Elytrigia elongata* (syn. *Th. elongatum*) is available (Linc, Sepsi and Molnár-Láng, 2012) alongside karyotypes of the J genome from *Th. bessarabicum* and the St genome from *Ps. spicata* (Linc *et al.*, 2017). A karyotype of the P genome of the *Agropyron* genus (a genus previously categorised with *Thinopyrum*) is not currently available.

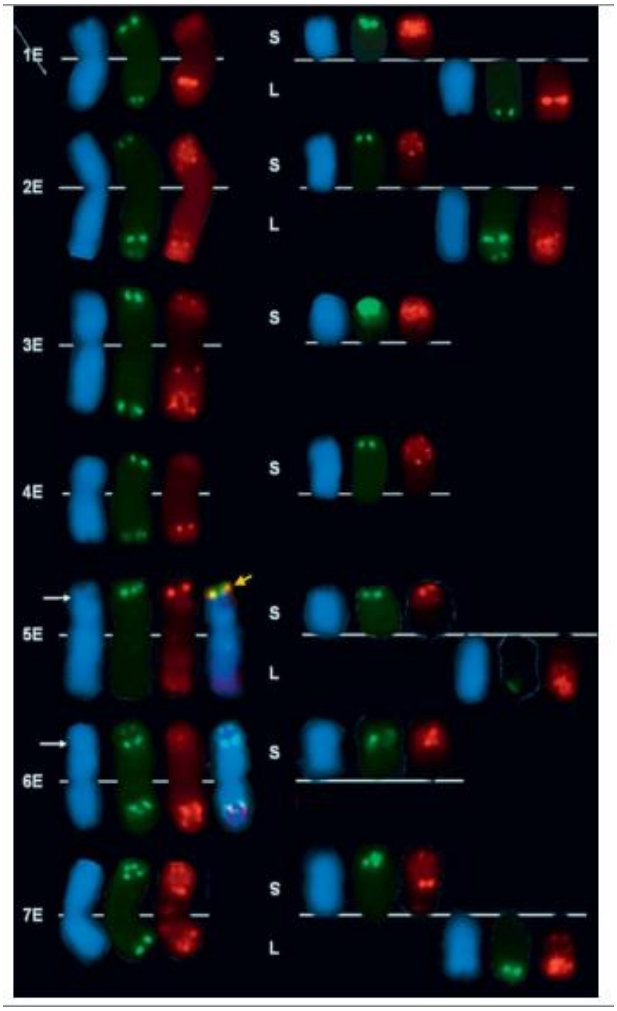


Figure 5.1: The FISH karyotype for the E genome presented in by Linc, Sepsi and Molnar-Lang (2012). Probes used include pSc119.2 (green), Afa family (red) and pTa71 (yellow).

The production of these karyotypes, alongside other molecular marker work confirmed the close relationship between the J and E genomes, and showed that the P genome of the *Agropyron* genus and St genome of *Pseudorogneria* are more distantly related (Linc *et al.*, 2017). This increased understanding of the genomic relationships will support future breeding programmes using the *Thinopyrum* genus to increase genetic variation in a wheat background.

When using FISH to detect introgressions, there is the possibility that small segments of introgressed wild relative material in a wheat background will not contain the specific sequence the FISH probe is designed to illuminate and so will not be detected. This is where GISH has the advantage (see section 5.1.3). It is possible that by using total genomic DNA, smaller segments can be detected in a wheat background. It should be noted that FISH and GISH can both miss the smallest of segments. FISH could fail to identify the

presence of a segment if it does not contain the corresponding sequence the probe is designed from. GISH could fail to identify a sequence if it is physically too small to be detected accurately. However, both GISH and FISH can be used to approximate introgression size and break points of segments can be visualised (Schwarzacher *et al*. 1992).

5.1.3. Genomic *in situ* hybridisation

Genomic *in situ* hybridisation (GISH) is a highly versatile molecular cytogenetic tool that emerged in the early 1990's as a modification of FISH (Silva and Souza, 2013). For GISH, fluorescent probes are directly bound to total genomic DNA from a target organism and used to probe corresponding chromosomes. Blocking DNA made from total genomic DNA of another organism is required to ensure the probe binds with a high specificity to just its target sequence. For example, in this research when visualising introgressions from wild relatives in wheat, the total genomic DNA of the wild relative used (*Th. elongatum* 401007) was extracted and directly bound to fluorescent probes. The total genomic DNA from wheat was extracted and sheared to make blocking DNA. When both blocking DNA and the selected probes were applied to microscope slides with preparations of hybrid metaphase spreads, the wild relative probe hybridised to any *Th. elongatum* chromosomes/introgressions and the blocking DNA bound to the corresponding wheat chromosomes, thus enabling the two organisms' genomes to be differentiated on a single slide.

Whilst FISH uses specific DNA sequences to probe samples, GISH uses total genomic DNA meaning the whole chromosomes hybridise with the probe rather than just specific sequences along the chromosomes. This makes GISH highly suitable for identifying additions and introgressions in hybrid lines. GISH has also being used extensively in progenitor studies to ascertain the origins and evolutions of allopolyploids such as wheat (Silva and Souza, 2013). Prior to the introduction of molecular cytogenetics, genome relationships were analysed using chromosome pairing studies, however results were variable and affected by genome homology. Due to the use of blocking DNA in GISH, the probes should only bind to a highly specific sequence, thus increasing the accuracy of identifying genome relationships. Whilst GISH cannot allow you to identify a specific sequence in a chromosome spread, it produces easy to analyse, visual results reliably.

Previous uses of GISH and FISH have been in identifying specific chromosomal locations of genes of interest. For example, FISH and GISH were used in a combination approach to identify the chromosomal location of the blue-grained gene (*Ba*). The *Ba* gene is a popular morphological marker from *Thinopyrum* species (see section 6.1.1). Back-crossed lines between wheat and *Agropyron ponticum* (syn. *Th. ponticum*) with this blue-grained physiological trait were analysed using FISH and GISH. The results showed that the phenotype mapped to the long arm of chromosomes 4Ag (called as such because at the time of the study the genome from which the chromosomes came from was unknown) from *Ag. ponticum* (Zheng *et al.*, 2006).

GISH can be limited by the homology of genomes – for example, the incredibly close relationship of the E and J genomes within the *Thinopyrum* genus means that a J genomic probe can bind to the E genome and vice versa. In addition to this, using total genomic DNA from a polyploid which contains multiple genomes will produce a probe that will bind to matching target sequences indiscriminately, for example a probe made from *Th. intermedium* (2n = 2x = 42, $JJJ^{St}J^{St}StSt$) would bind to any J or St genome sequences.

Single - colour GISH (sc-GISH) is a higher-throughput method than FISH, more suited for screening back-crossed populations for wild relative introgressions, however FISH can potentially identify from which chromosome the introgression originates. This is dependent on the size of the introgression being analysed, the distinctiveness of the banding pattern produced and the availability of a karyotype. The throughput of GISH lowers with each probe being analysed on the same sample, so sc-GISH is higher throughput than multi-colour GISH (mc-GISH), hence why it is more suitable for identifying the presence of introgressions in a large back-crossed population. An excellent review of the use of GISH to study and improve crop species is Silva and Souza (2013) and references within. To summarise the difference between FISH and GISH, FISH is most suitable when researchers want to detect the presence of a specific sequence, whereas GISH is most suitable when researchers want to detect the presence of a specific genome. As this research is focussed on detecting the presence of any DNA from *Th. elongatum* within a wheat background, GISH is the more appropriate technique.

5.1.4. Multi-coloured cytogenetic techniques

Multi-coloured FISH (mc-FISH) and multi-coloured GISH (mc-GISH) were introduced in the early 1990's and increased the power of single coloured GISH and FISH by binding multiple probes to a sample simultaneously (Mukai, Nakahara and Yamamoto, 1993; Fukai, Ohmido and Khush, 1994). By using multiple probes at once, it became possible to discriminate between genomes simultaneously, to physically order sequences on chromosomes for mapping and to identify multiple progenitors of allopolyploid species (Singh, 2003).

Mc-FISH has been used to produce karyotypes for the E, J, and St genomes. The use of mc-FISH also showed differences between individual accessions of the same species. Interestingly, there was greater variation in the *Th. elongatum* accession karyotypes than the *Th. bessarabicum* accession karyotypes (Linc *et al.*, 2017).

Mc-GISH can be particularly useful when investigating phylogenetic relationships of species, or for identifying introgressions within allopolyploid species. For example, mc-GISH and subsequent genomic southern hybridisation were used to show that the St and E genomes of the *Thinopyrum* genus are most closely related to the D genome of wheat, then the A genome and most distantly to the B genome (Liu, Li and Zhang, 2007). This could have suggested that most wheat/*Th. elongatum* introgressions produced in this study will be found in the D genome of wheat, then the A and most rarely in the B genome. However, recent research is now suggesting that despite the D and E genomes being closely related, recombination events are more frequently occurring than expected between the B , A and E genomes (Grewal *et al.*, 2018). Therefore, these genomic relationships may need reconsidering and introgressions from *Th. elongatum* may be found more frequently than expected within the A and B genomes of wheat.

Mc-GISH was the technique used to discriminate the three genomes within the allopolyploid wheat (cv. Chinese Spring) by the use of biotinylated total genomic DNA from *T. urartu* (A genome), digoxigenin-labelled total genomic DNA from *Ae. tauschii* (D genome) and non-labelled DNA from *Ae. speltoides* (B genome) (Mukai, Nakahara and Yamamoto, 1993). This advance allowed introgressions in hybrid lines to be characterised in greater detail, and

enabled the relationships between genomes to be studied. As with sc-GISH, mc-GISH can be limited by the homology of the genomes being analysed, the E and J genomes have proven particularly challenging to differentiate between (Zhang, Dong and Wang, 1996).

More recently, the use of mc-GISH has shown that in back-crossing programmes intergenomic recombination events are not just limited to the genomes of wheat exchanging chromatin with the wild relative genome, but can also happen between the A, B and D genomes of wheat (King *et al.*, 2017)

Although the *in situ* hybridisation techniques are powerful and multi-functional, they are time-consuming, laborious and can be limited in the overall information gained about hybrid lines. However, when combined with molecular markers, they provide a more thorough insight into hybrid lines produced whilst helping the development and validation of molecular markers for future high-throughput introgression detection in hybrid lines.

5.1.5. A combined cytogenetic and molecular approach

When cytogenetic techniques are used in collaboration with molecular markers a more complete picture is obtained for individual lines. For example, a wide range of cytogenetic and molecular tools were used to characterise diploid *Th. elongatum* addition lines with increased resistance to FHB (Liu *et al.*, 2017). However, the utilisation of a combined cytogenetic and molecular approach enabled the researchers not only to identify addition lines but also to locate FHB resistance on chromosome 1E for the first time (Jauhar, Peterson and Xu, 2009).

GISH, mc-GISH and mc-FISH were initially utilised to characterise 12 wheat - *Th. bessarabicum* recombinant lines. These recombinant lines were then further characterised using SNP markers and a total of 1150 SNP markers were physically mapped to the *Th. bessarabicum* J genome. These lines and SNP markers are now powerful tools for future work detecting introgressions from *Thinopyrum* or from species that contain closely-related genomes (Grewal *et al.*, 2018).

The utilisation of a combined approach of cytogenetic and molecular markers increases the reliability of identifying and characterising introgressions. In

previous research, the use of sc-GISH showed a whole *Th. elongatum* addition line, however subsequent analysis using SNP markers actually revealed the whole chromosome was a 6E/7E recombinant chromosome (Lou *et al.*, 2017).

This research will use a cytogenetic approach to detect initial introgressions and attempt to develop molecular markers to confirm findings. Any molecular markers produced would become highly useful and novel tools for accurate, high-throughput detection of introgressions from *Th. elongatum* 401007 in future wheat hybrid lines.

5.2. Aims and Objectives

The aims and objectives of this chapter are:

- Analyse as many advanced lines from the *Th. elongatum* 401007 breeding programme using single colour GISH to identify lines with single introgressions and homozygous introgressions.
- Investigate *Th. elongatum* 401007 progenitor species using multi-colour GISH.
- Investigate the genomic relationships in introgression lines by using multi-colour GISH to see if there is a preferential transfer of *Th. elongatum* chromatin to the A, B or D genome of wheat.
- Investigate the genomic relationships in introgression lines by using multi-colour GISH to see if there is a preferential transfer of E(J) or E^{St} (J^{St}) genomes from *Th. elongatum* 401007 into wheat.

5.3. Materials and Methods

5.3.1. Root tip collection and enzyme digestion

De-ionised water was sprayed onto germinating seedlings daily whilst being monitored for radicle emergence (see section 4.3.3 for germination). Once the roots had emerged from the radicle and grown to a length of approximately 1.5cm the actively growing region was harvested using forceps. The seedlings were then planted up in trays in Levington Seed and Modular compost (F2+S) for a week to establish themselves before vernalisation (see section 4.3.4).

Once harvested, the root tips were placed into pre-labelled 0.5ml Eppendorf tubes and sprayed with de-ionised water to prevent cell shrinkage. The lids of the Eppendorf tubes were pierced and then placed into a nitrous oxide chamber (see Figure 5.2).

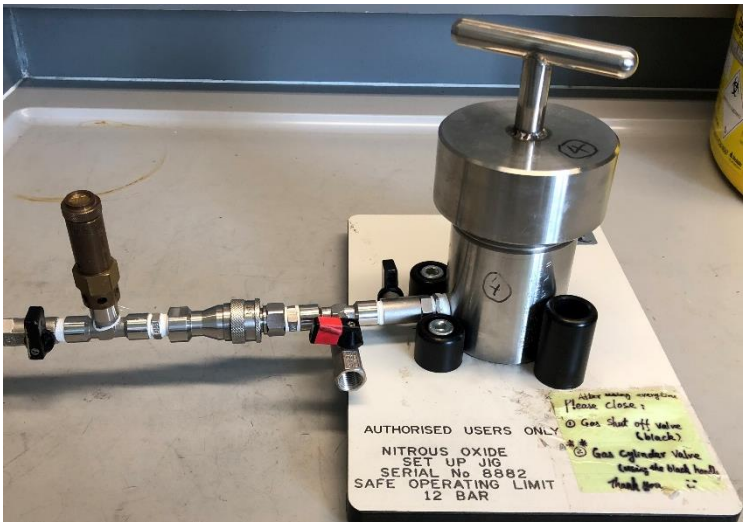


Figure 5.2: The nitrous oxide chamber

Nitrous oxide gas was released into the chamber at a pressure of 10 bar for 30 seconds. The chamber was sealed and samples were kept in the chamber for 2 hours. Once removed from the chamber, each Eppendorf tube was filled with 300µl of 90% acetic acid for 10 minutes and then washed in de-ionised water three times**.**

Once washed, root tips underwent nitrous oxide- enzymatic maceration (adapted from Kato, Lamb and Birchler, 2004). After washing, individual root tips were placed on filter paper to remove excess water before the actively dividing section of the root was removed using a scalpel and transferred to a

0.5ml labelled Eppendorf tube containing 20µl of 1% pectolyase Y23 and 2% cellulose Onozuka R-10 (Yakult Pharmaceutical, Tokyo) enzyme solution.

Tubes were sealed and incubated at 37ºC for 50-54 minutes, depending upon the width of the root tips and the generation of the backcross being digested. Immediately after incubation tubes were cooled using ice and samples were washed with 70% ethanol three times. Root tips were then stored at -20ºC in 100µl of 70% ethanol or immediately taken forward to the metaphase chromosome spread production.

5.3.2. Metaphase chromosome spread preparation

Digested root tips suspended in 100µl of 70% ethanol were gently crushed using a dissection needle to form a cell suspension. Once the sample was fully suspended, tubes were centrifuged for 2 minutes at 6000rpm. The supernatant was carefully removed without disturbing the pellet and the pellets were air-dried at room temperature for approximately 5 minutes. Cells were resuspended in 15µl of 100% acetic acid and left on ice for 1 hour.

Thermo Scientific (D-38116, 76 x 26mm) slides were labelled with the relevant plant ID and placed in a cardboard box lined with layers of damp tissue over Clingfilm. Tubes were tapped to suspend cells in the 100% acetic acid. 7µl of cell suspension was dropped on to the matching labelled slide from a height of approximately 5cm. The cardboard box was kept closed as much as possible to prevent slides drying out too quickly. Once dry, slides were stored at 4ºC for use within one month, or at -20ºC for later use.

5.3.3. Purified DNA extractions for probes

DNA was extracted from leaf material as outlined in section 3.2. Any DNA extracted for use in genomic *in situ* hybridisation required an additional phenol chloroform step to purify the DNA. After the pellets were re-suspended in 100 – 200µl of de-ionised H_2O, 200µl of phenol chloroform was added to each tube. Samples were mixed until cloudy and then centrifuged at 13,000rpm for 5 minutes. The supernatant from each sample was transferred to a new tube to which 20µl of 3M NaAC (pH 5.2) (Appendix 9.1) and 500µl of 100% ethanol were added. Samples were left at -20ºC overnight.

Tubes were centrifuged for 10 minutes at 13,000rpm to pellet the DNA, the supernatant removed and the pellets were washed in 70% ethanol three

times before air drying. Once air-dried, pellets were suspended in 100-200µl de-ionised H_2O, left at 4ºC for 1 hour and vortexed. Samples then had their concentrations measured (section 3.3) and were stored at -20ºC.

5.3.4. Genomic DNA probe production

Nick translation was used to produce hybridisation probes from total genomic DNA extracted from the following wild relatives:

1. *Th. elongatum* 401007
2. *Th. elongatum* 531718
3. *Th. bessarabicum*
4. *Ps. stipofolia*
5. *Ae. tauschii*
6. *T. urartu*
7. *D. villosum*
8. *Ae. speltoides*
9. *S. cereale*

For each nick translation reaction the following reagents were added to a 1.5ml Eppendorf tube:

- 2µl total genomic DNA (concentration 1000ng/µl)
- 2µl - 10 x Nick translation buffer (New England Biolabs Ltd)
- 2µl - non-labelled dNTPs (2mM each, mixed)
- 0.5µl - labelled dNTP (1mM) (Life Technologies™)
- 5µl - DNA Polymerase I (M0209L, New England Biolabs Ltd)
- 0.8µl - DNase (100mU/µl)
- 7.7µl dH_2O

Genomic DNA from *Th. elongatum* 401007, *Th. elongatum* 531718, *Th. bessarabicum, Ae speltoides, D. villosum* and *T. urartu* was labelled by nick translation with Chroma Tide Alexa Fluor 488-5-dUTP (Life Technologies™; C11397). Genomic DNA from *Ps. stipofolia* and *Ae. tauschii* was labelled by nick translation with Chroma Tide Alexa Fluor 594-5-dUTP (Invitogen; C11400). Genomic DNA from *Th. elongatum* 531718, *Th. elongatum* 401007 and *S. cereale* was labelled by nick translation with Chroma Tide Alexa Fluor 546-14-dUTP (Life Technologies™; C11401).

Tubes were mixed thoroughly via pipette and incubated for 2 hours at 16ºC in the dark. After incubation, 160µl of 140ng/µl SSDNA was added to each

Eppendorf tube and vortexed. 500µl of 9:1 precipitation solution (V/V 100% ethanol: 3M sodium acetate pH 5.2) (Appendix 9.1) was then added to each Eppendorf and vortexed before samples were left overnight in the -20ºC freezer to precipitate.

Tubes were centrifuged at 12,000rpm for 30 minutes at 4ºC. The supernatant was removed and the probe pellets were washed in 300µl of 70% ethanol and centrifuged for 5 minutes at 4ºC before the supernatant was removed again. This wash step was repeated once more. Probe pellets were air dried for 10 minutes and once all traces of ethanol were gone, pellets were dissolved in 20µl of 2xSSC+1xTE buffer (pH 7.6), left on ice for 30 minutes, mixed by gently tapping and then stored at -20ºC.

5.3.5. Genomic blocking DNA preparation

700µl of purified total genomic wheat DNA (see Chapter 5.3.3) was aliquoted into a labelled 1.5ml Eppendorf tube and sealed tightly with several layers of Clingfilm. The DNA was then fragmented to 300-500 base pairs in length by heating in a heat block set to 110ºC for 15 minutes. Fragmented DNA was then stored at -20ºC.

5.3.6. Protocol for detecting metaphase chromosome spreads

Slides of metaphase spreads (see section 5.3.2) were cross-linked with UV at 0.125 Joules twice. The following probe mixture was added to each slide for sc-GISH:

- 1µl of *Th. elongatum* 401007 genomic DNA probe (200ng/µl)
- 2µl of fragmented blocking wheat DNA (2000ng/µl)
- 7µl of 2 x SSC in 1 x TE buffer (Appendix 9.1)

10µl of this mixture was added to each slide and a plastic cover slip (22mm x 22mm) was applied carefully to avoid air bubbles. Slides were placed on wet tissue in a stainless steel tray and placed in a water bath (70-75ºC) for 5 minutes in the dark. Slides were transferred to a container with a slide holder and wet tissue inside. Slides were incubated at 55ºC overnight.

Slide covers were removed by dipping each slide into 2x SSC. One drop of Vectashield mounting medium with the counterstain DAPI dihydrochloride (1:1 diluted with 1xPhosphate Buffered Saline) was dropped on each slide before a glass cover slip was placed on top, again avoiding air bubbles.

Slides were observed using a multi-filter microscope (Axio Imager.Z2, Zeiss, Germany). Filters used were for DAPI (blue), Alexa Fluor 488 (green) and Alexa Fluor 594 (red). Results were analysed using the Isis FISH Imaging System V5.7 (MetaSystems GmbH, Germany).

For mc-GISH analysis the probe mixture varied. Twenty-nine lines were probed with total genomic DNA from the A, B and D genomes of wheat. The probe mixture for each of these slides was as follows:

- 1.2µl of A genomic probe
- 4.0µl of B blocking DNA
- 0.8µl of D genomic probe
- 1.0µl of *Th. elongatum* 401007 genomic probe
- 3.0µl of 2xSSC in 1xTE

Another 32 lines were probed with total genomic DNA from the J and St genomes found in *Thinopyrum* species. The probe mixture for each of these slides was as follows:

- 1µl of J genomic probe
- 1µl of St genomic probe
- 3µl of Chinese Spring blocking DNA
- 5µl of 2xSSC in 1xTE

All probes used in this section were produced as outlined in section 5.3.4 and the blocking DNA was produced as outlined in section 5.3.5.

5.4. Results

5.4.1. Single-colour GISH

To date, 372 lines have been digested to produce slides with metaphase spreads and 327 of these lines have been characterised using sc-GISH. A total of 143 introgressions and 660 whole *Th. elongatum* chromosomes were visualised from 119 individual lines. At the BC_1 generation, 3 lines contained a total of 4 introgressions and 26 whole *Th. elongatum* chromosomes. Fourteen BC_2 lines contained 7 introgressions and 101 whole *Th. elongatum* chromosomes. Seventy-six BC_3 lines contained 30 introgressions and 234 whole *Th. elongatum* chromosomes. Twenty-nine BC_3F_1 lines contained 11 introgressions and 74 whole *Th. elongatum* chromosomes. Forty-three BC_4 lines contained 21 introgressions and 60 whole *Th. elongatum* chromosomes. Eighty-six BC_4F_1 lines were shown to have 57 introgressions and 100 whole *Th. elongatum* chromosomes. At the BC_5 generation 66 lines were shown to contain 12 introgressions and 49 whole *Th. elongatum* chromosomes. Finally at the BC_5F_1 generation, 10 lines contained 1 introgression and 16 whole *Th. elongatum* chromosomes. Table 5.1 contains a summary of the number of lines that underwent the sc-GISH method and the results obtained from these experiments.

Table 5.1: A summary of the number of lines that underwent the sc-GISH protocol, the number of lines that were successful and the number of lines that contain introgressions. The total numbers of introgressions produced are also included.

	Number of lines GISHed	Number of successful GISHes	Number of GISHed lines with introgressions	Total number of introgressions visualised per generation
BC_1	3	3	3	4
BC_2	14	14	6	7
BC_3	83	76	28	30
BC_3F_1	37	29	9	11
BC_4	51	43	17	21
BC_4F_1	94	86	44	57
BC_5	78	66	11	12
BC_5F_1	12	10	1	1
Totals	**372**	**327**	**119**	**143**

Table 5.2 contains results showing an analysis of the germplasm production, including the percentages of lines that were shown to have an introgression at each generation, the average number of introgressions and whole chromosomes per line in each generation and the total number of whole *Th. elongatum* chromosomes transferred to each generation. If any lines had homozygous introgressions, those introgressions were only counted once.

Table 5.2: A summary of GISHed lines, showing the average number of introgressions present in each generation, the total number of whole chromosomes transferred from *Th. elongatum* 401007 and averages.

	Percentage of lines GISHed with an introgression (%)	Average number of introgressions per line	Total number of whole *Th. elongatum* chromosomes transferred	Average number of whole *Th. elongatum* chromosomes per line
BC_1	100	1.33	26	8.67
BC_2	42.9	0.50	101	7.21
BC_3	36.8	0.39	234	3.08
BC_3F_1	31.0	0.38	74	2.55
BC_4	39.5	0.49	60	1.40
BC_4F_1	51.2	0.66	100	1.16
BC_5	16.7	0.18	49	0.74
BC_5F_1	10	0.10	16	1.60

Figure 5.3 shows a selection of sc-GISH images from a range of generations in the wheat/ *Th. elongatum* 401007 breeding cycle. Images shown display the wide range of variation in introgression size, whole chromosome number transferred and total number of chromosomes seen throughout the crossing programme.

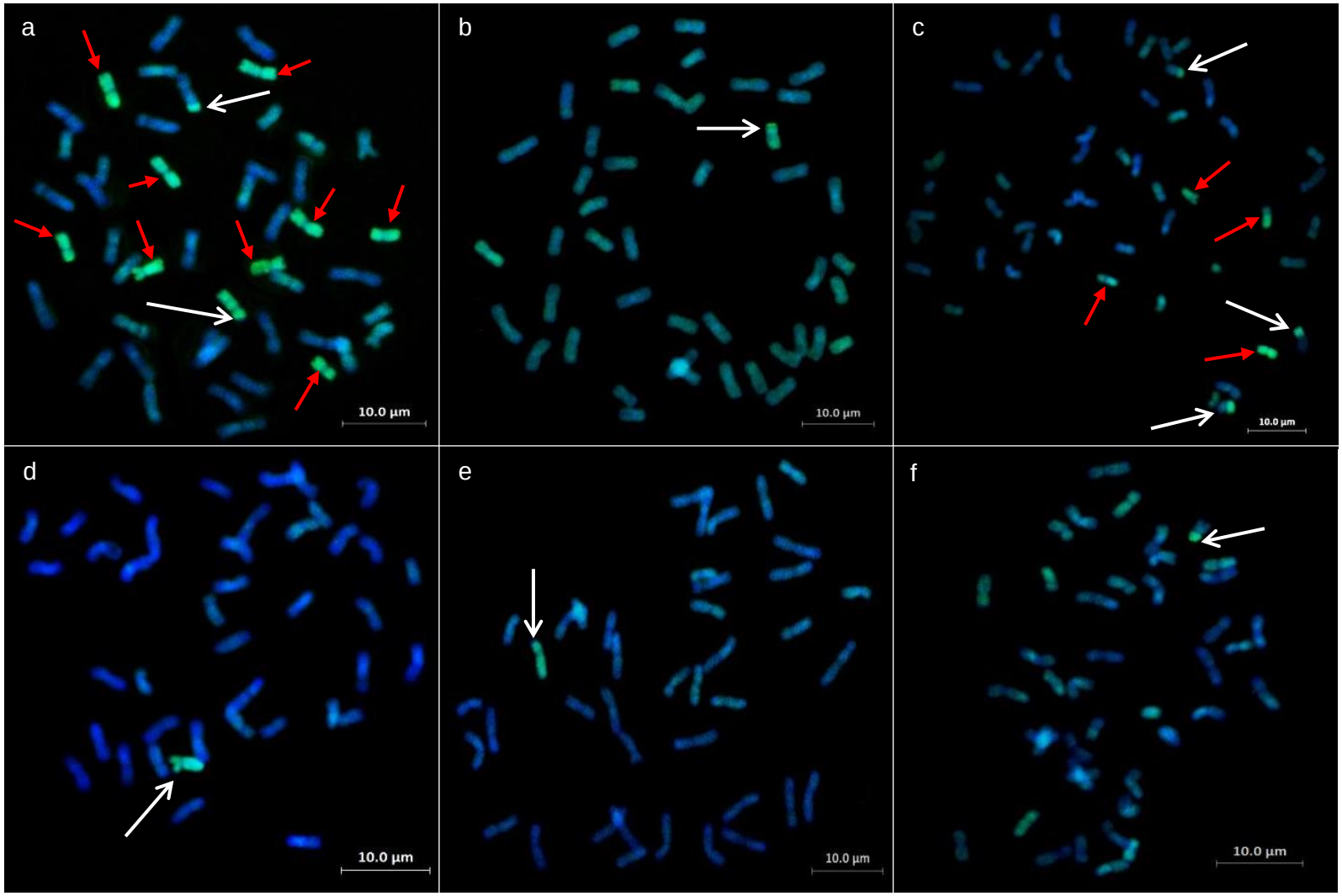


Figure 5.3: A selection of sc-GISH images of metaphase spreads. All spreads were stained with fluorescently labelled total genomic DNA from *Th. elongatum* 401007 (green) and total fragmented genomic DNA from Chinese Spring was used as blocking DNA (blue). Introgressions are indicated using white arrows. Whole *Th. elongatum* chromosomes are indicated using red arrows. (a) BC_2-741D (9 whole and 2 introgressions), (b) BC_3-670C (single introgression), (c) BC_3F_1-229A (3 introgressions, 4 whole and a centromeric break), (d) BC_4-179D (single large introgression), (e) BC_4F_1-101D (single large introgression) and (f) BC_5F_1-4A (single introgression).

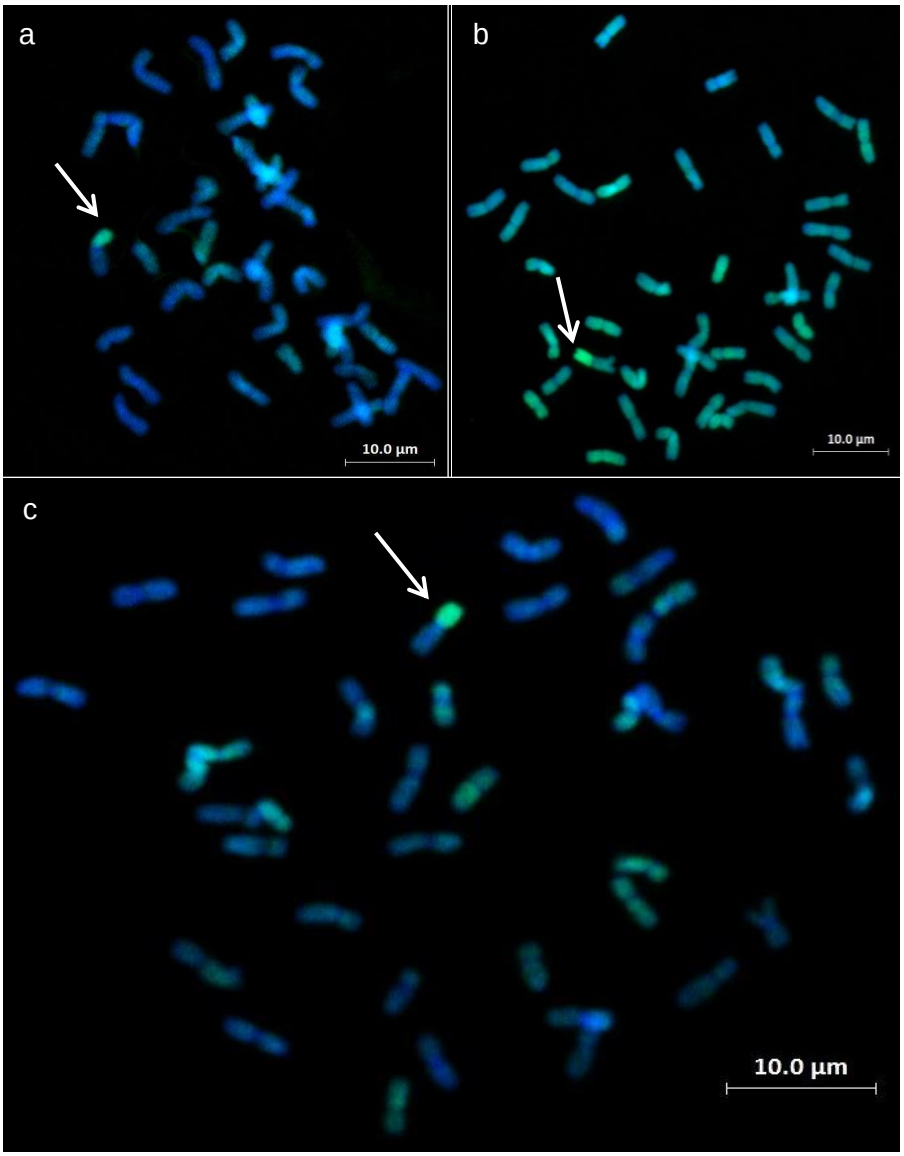


Figure 5.4 (a) BC_4-172A, (b) BC_4-172B and (c) BC_4-172C, all probed using fluorescently labelled total genomic DNA from *Th. elongatum* (green) and blocking DNA of Chinese Spring (blue). All three lines from the same family appear to have the same single introgression present, indicated using white arrows.

Figure 5.4 shows the sc-GISH results of three individual plants from the same family. The BC_4-172 lines were produced from BC_3-151B, which came from BC_2-229A, which came from BC_1-196H. No GISH images are available from previous lines in this particular family tree. Without more data from other families, or from molecular markers or mc-GISH, it is not possible to state whether specific introgressions are being preferentially transferred, or whether this observation is coincidental. In other families, 'sibling' lines appear to have possible reciprocal introgressions, possibly due to the recombination of chromatin during meiosis. Figure 5.5 shows such an

occurrence, with the line BC_4-179A having a small introgression at the telomere, and its 'sibling' BC_4-179C having a large introgression, with only the telomere being wheat chromatin.

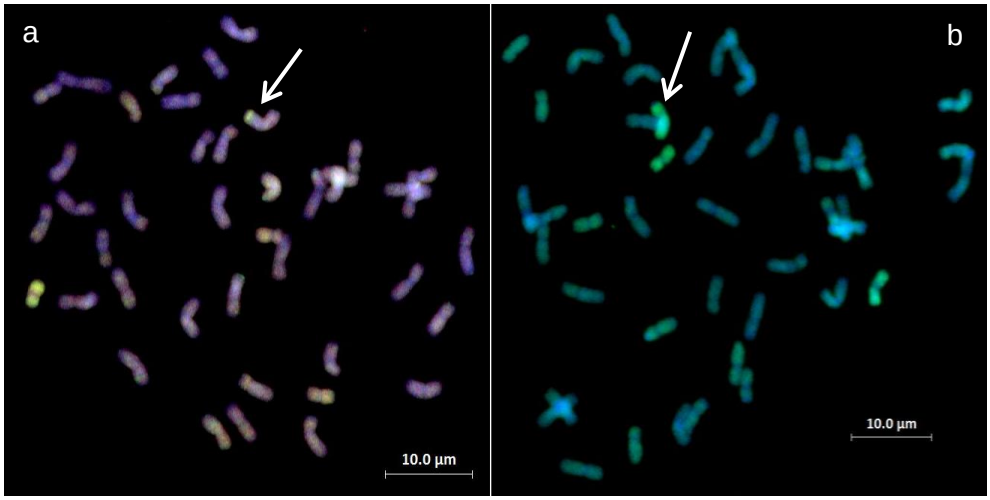


Figure 5.5: The mcGISH image (a) from BC_4-179A shows one whole chromosome and a very small introgression; and the sc-GISH (b) image from the sibling line BC_4-179C shows two whole chromosomes and one very large introgression. These 'sibling' lines appear to be showing apparent reciprocal introgressions. The introgressions are indicated using white arrows. The mc-GISH slide was probed with fluorescently labelled total genomic DNA from *Th. bessarabicum* (green) and *Ps. stipofolia* (red). The sc-GISH slide was probed with fluorescently labelled total genomic DNA from *Th. elongatum* 401007 (green). Both slides used fragmented total genomic DNA from Chinese Spring as blocking DNA.

In order to further characterise the introgressions being identified from sc-GISH, a mc-GISH approach was undertaken. The A, B and D genomic DNA probes were used to investigate which genomes of wheat the introgressions from *Th. elongatum* were recombining with. Total genomic DNA probes of the J and St genomes were used to investigate whether any of the *Th. elongatum* 401007 genomes were being preferentially transferred into wheat.

5.4.2. Multi-colour GISH

Twenty-nine lines which had undergone sc-GISH were selected from available materials to undergo mc-GISH with probes of the A, B and D genomes of wheat. These 29 lines were found to contain 32 introgressions. Of these lines, 100% of the mc-GISH results matched their corresponding sc-GISH. Table 5.3 shows with which genome of wheat the *Th. elongatum* introgression recombined with.

Table 5.3: The multi-colour GISH results grouped according to which genome of wheat the introgressions from *Th. elongatum* 401007 recombine with.

Recombinant Type	Number of Introgressions
Single Recombinants	
E with A	13
E with B	11
E with D	4
Double Recombinants	
E with one D telomere and one A telomere	1
E with two D telomeres	1
E telomeres with a D centromere	1
E telomere with an A centromere and D telomere	1
Total	**32**

Figure 5.6 and Figure 5.7 show mc-GISH results for two different lines from the wheat/ *Th. elongatum* 401007 breeding programme. Both figures include two images of the same metaphase spread as it was not possible to display

the green A (*T. urartu*) probe and the gold E (*Th. elongatum*) probe simultaneously. This is because both probes appeared as green under the microscope filter required and were therefore difficult to differentiate between on the same image.

Figure 5.6 shows the mc-GISH results from BC_4F_1-95D. The previous results from the sc-GISH showed a single large introgression and one whole chromosome from *Th. elongatum* 401007. The mc-GISH revealed that the whole chromosome has a segment of D genome, revealing it is actually a recombinant chromosome between the E and D genome of wheat. The large introgression is shown to be a large E segment with a B genome telomere.

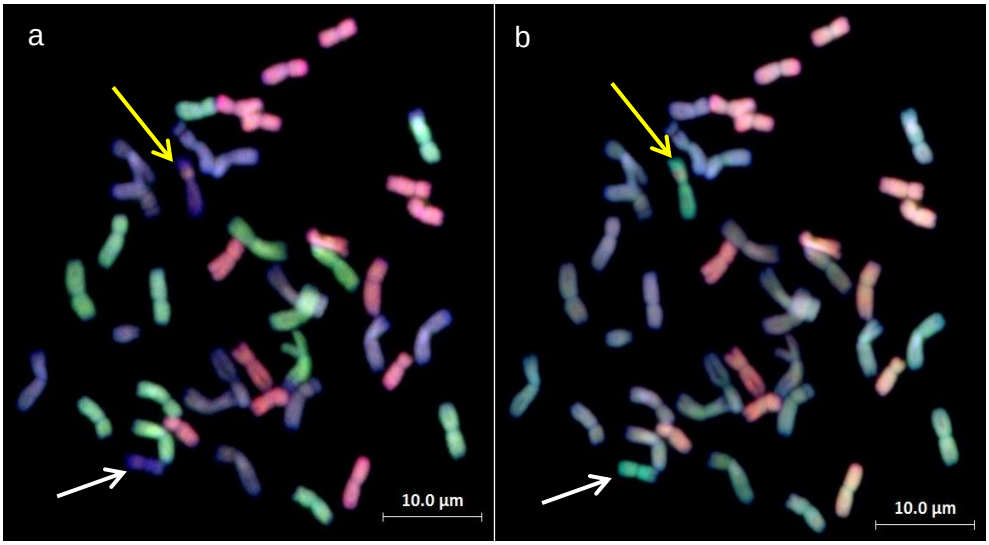


Figure 5.6: Two images of the same metaphase spread from BC_4F_1-95D. (a) Shows the spread probed with fluorescently labelled total genomic DNA from *Aegilops tauschii* (red) and *Triticum urartu* (green). Total genomic DNA from Chinese Spring was used as blocking DNA (blue). (b) Shows the same spread probed with *Aegilops tauschii* (red) and *Thinopyrum elongatum* 401007 (green), again using total genomic DNA from Chinese Spring as blocking DNA. The yellow arrows indicate the E-D whole chromosome and the white arrows indicate the large E-B introgression.

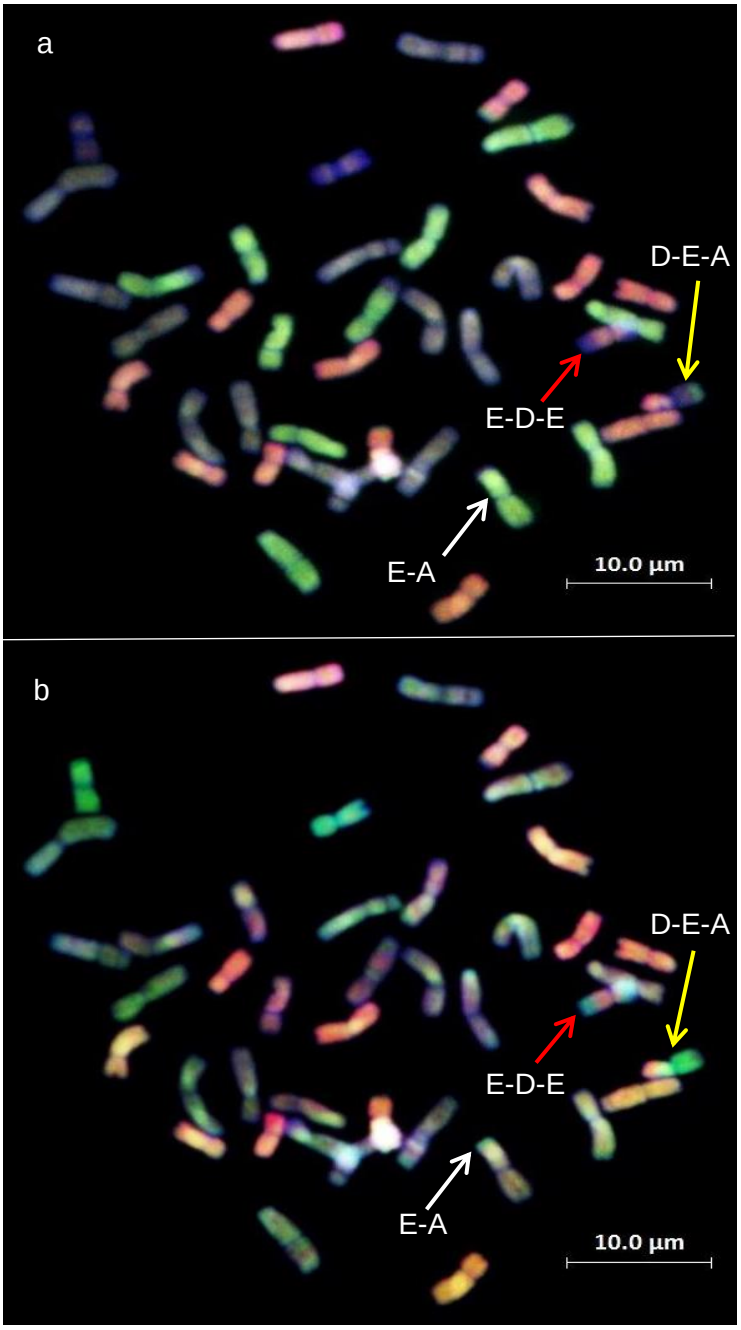


Figure 5.7: Two images of the same metaphase spread from BC_4-247A. Image (a) shows the A (*Triticum urartu*), B (Chinese Spring blocking DNA) and D (*Aegilops tauschii*) genomes (green, blue and red respectively), Image (b) shows the B, D and E (*Th. elongatum*) genomes (blue, red and green respectively). Both images show the B genome as blue due to being counter-stained with DAPI. The small E-A introgression is indicated using a white arrow. The E-D-E introgression is indicated using a red arrow and the D-E-A introgression is indicated using a yellow arrow.

Figure 5.7 shows the mc-GISH results of BC_4-247A. The previous sc-GISH results of this line indicated three different size introgressions and three whole chromosomes from *Th. elongatum* 401007 (data not shown). The mc-GISH results revealed that the smallest introgression (white arrow) was between the

E genome and A genome of wheat. The second introgression (red arrow) was between the E genome and D genome, where both telemetric regions were comprised of the E genome and the centromeric region was from the D genome of wheat. The final introgression (yellow arrow) was shown to have a D telemetric region, a large E centromeric region and an A telemetric region.

5.4.2.1. Mc-GISH of the wild relatives

Due to the difficulty of classifying the genomes in the *Thinopyrum* genus an independent mc-GISH experiment was undertaken to investigate whether the accession *Th. elongatum* 401007 was an autopolyploid or an allopolyploid and to identify potential progenitor species.

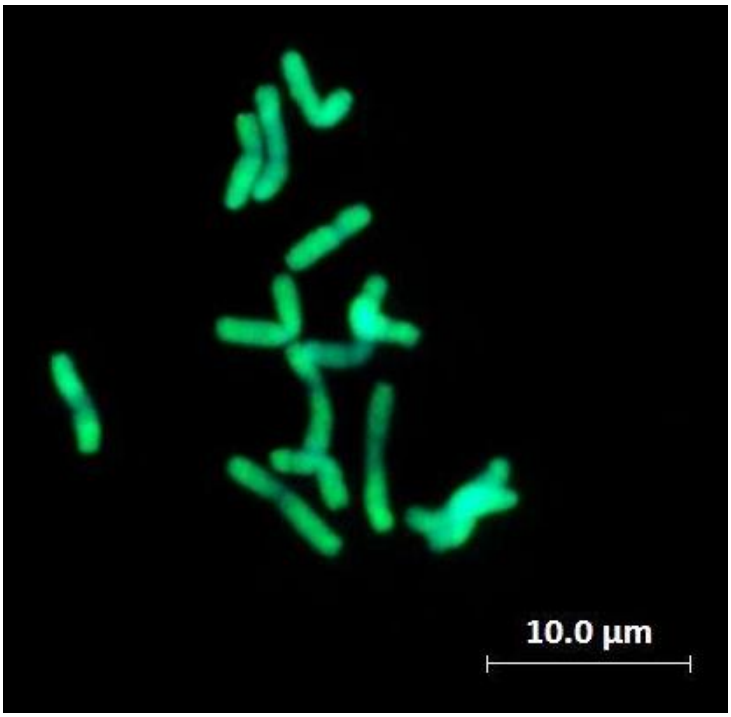


Figure 5.8: A metaphase spread from *Th. elongatum* 531718 (2n = 2x = 14) probed with fluorescently labelled total genomic DNA from *Th. bessarabicum* (genome = JJ). These results show that the J genome clearly binds with the chromosomes. Counter-stained with DAPI.

The diploid accession of *Th. elongatum* (acc. 531718) was investigated first. Two high quality slides containing metaphase spreads from *Th. elongatum* 531718 were produced as outlined in section 5.3.2. The first slide was probed with total genomic DNA from itself, *Th. elongatum* 531718, as a control (E genome – image not available). The second slide was probed with total genomic DNA from *Th. bessarabicum* 531711 (genome = JJ, Figure 5.8) to investigate the homology of the E and J genomes.

A third high quality slide was probed with a St genomic probe from *Ps. stipofolia* and a B genomic probe from *Ae. speltoides* (Figure 5.9).

The results show that the diploid *Th. elongatum* bound clearly with both the E genomic probe and the J genomic probe, supporting the high homogeneity between the E and J genomes already acknowledged in many studies. The St and B genomic probes bound strongly at telemetric sites in the diploid. However, this probably indicates the presence of conserved sequences, not that the B or St genome is present in the diploid *Th. elongatum* 531718 genome.

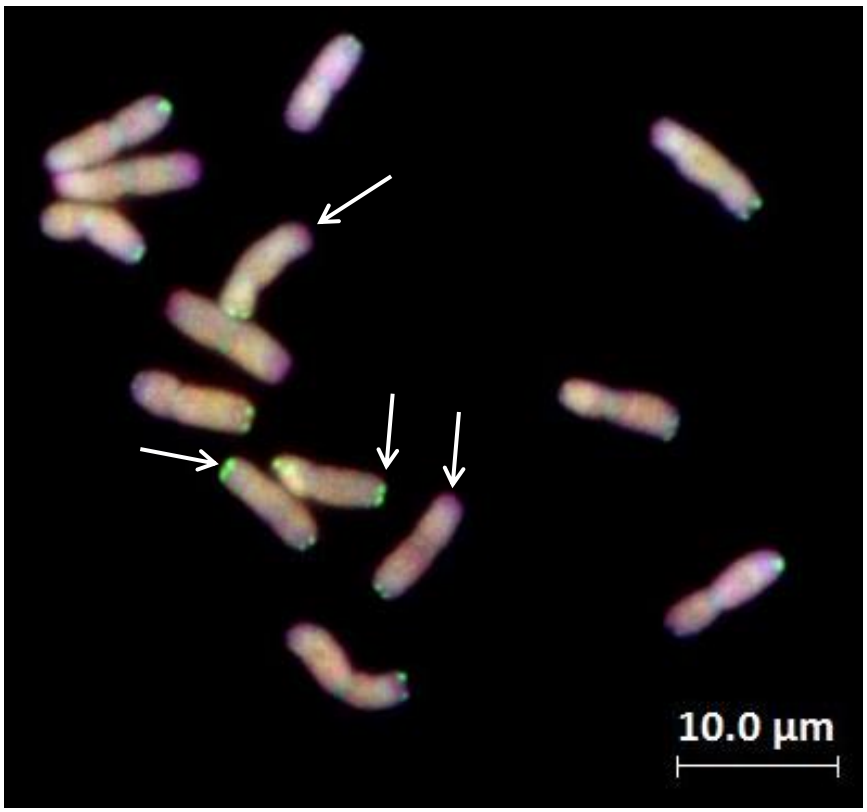


Figure 5.9: A metaphase spread from Th. elongatum 531718 probed with fluorescently labelled total genomic DNA from *Pseudorogneria stipofolia* (St genome - red probe) and *Aegilops speltoides* (B genome - green probe). Neither probes successfully hybridised to the diploid *Th. elongatum* chromosomes, except for at localised telemetric regions, most likely indicating the presence of a highly conserved sequence. Such regions are indicated using white arrows. No blocking DNA was used. Counter-stained with DAPI.

The same genomic probes were used to investigate the genomic composition of decaploid *Th. elongatum* 401007. *Th. elongatum* 401007 was also probed with a V genome probe from *D.villosum* due to its presence within the polyploid *Th. intermedium*. Figure 5.10 shows a chromosome metaphase spread from *Th. elongatum* 401007 (2n = 10x = 70+2) that has been probed

with *D. villosum* (genome = VV). The results indicate the presence of a conserved sequence in the telemetric regions – however there are no V genome segments present, therefore the V genome is not a progenitor of the decaploid *Th. elongatum*.

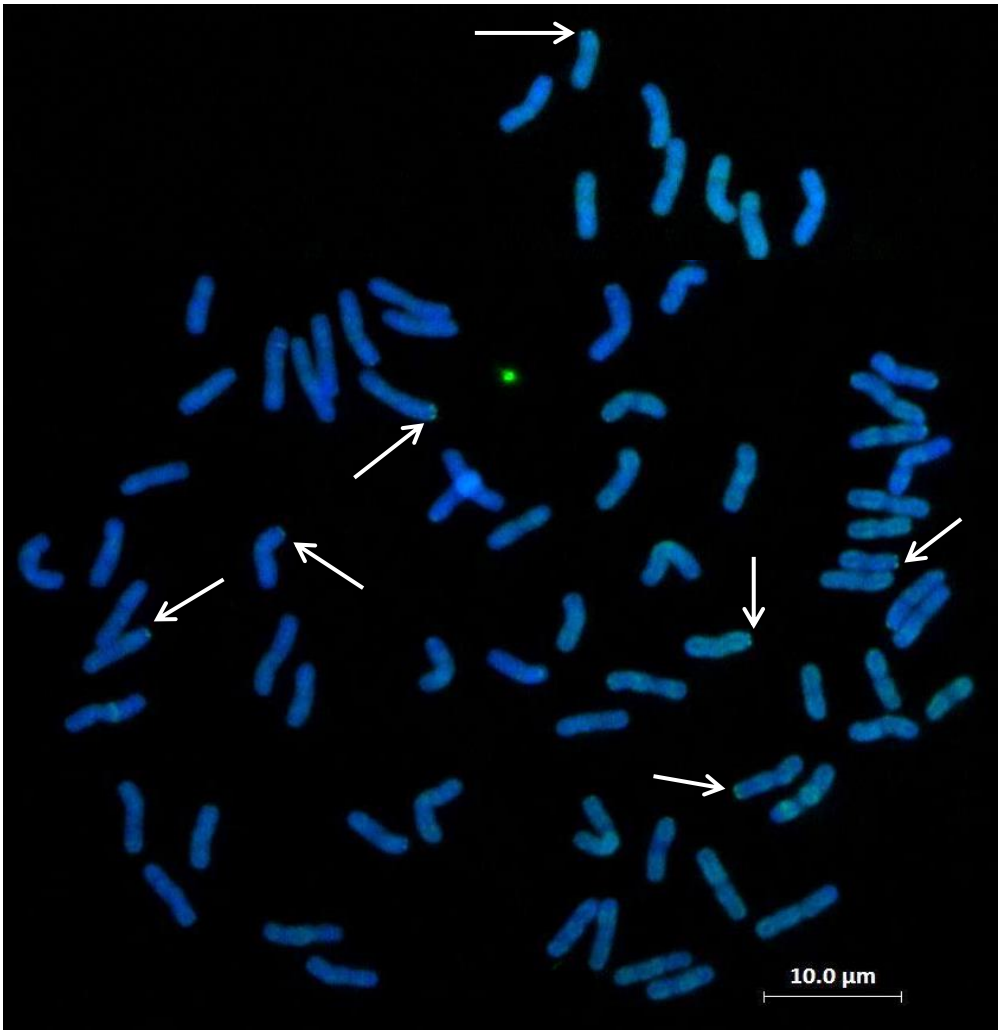


Figure 5.10: A metaphase spread of all 72 chromosomes in *Th. elongatum* 401007 probed with fluorescently labelled total genomic DNA from *D. villosum* (green) and counter-stained with DAPI (blue). There is no hybridisation of the V probe to the chromosomes of *Th.* elongatum 401007, except for conserved sequences in the telometric regions, which are indicated with white arrows. No blocking DNA was used.

A separate slide containing metaphase spreads from *Th. elongatum* 401007 was probed using total genomic DNA from *Th. bessarabicum* (genome = JJ) and *Ps. stipofolia* (genome = StSt). Another identically produced slide was probed with total genomic DNA from *Th. elongatum* 531718 (genome = EE) and *Ps. stipofolia*. Both results are shown in Figure 5.11.

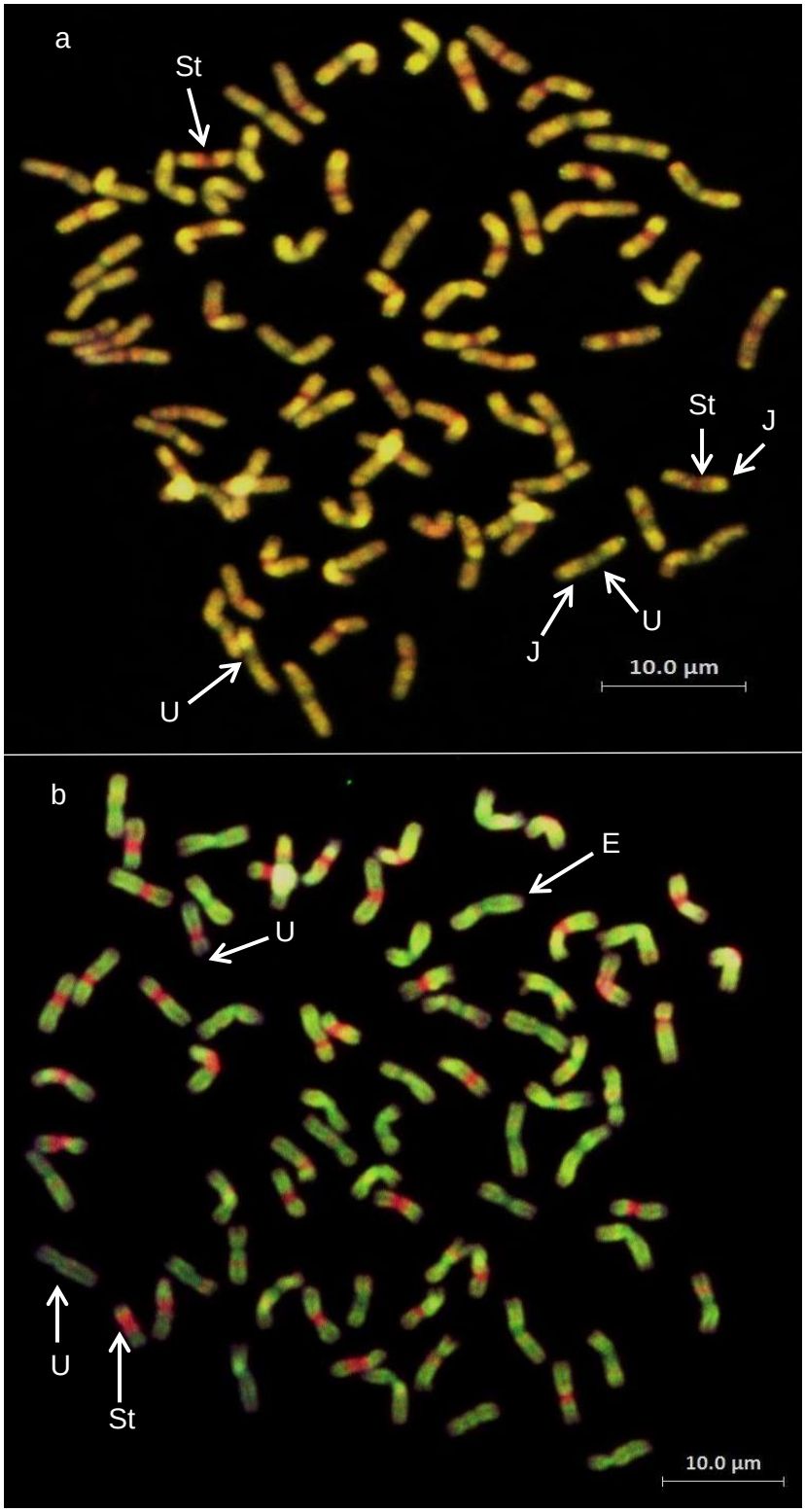


Figure 5.11: Metaphase spreads of *Th. elongatum* 401007 probed with fluorescently labelled DNA from (a) *Th. bessarabicum* (genome = JJ, gold) and *Ps. stipofolia* (genome = StSt, red); and (b) probed with *Th. elongatum* 531718 (genome = EE, green) and *Ps. stipofolia* (genome = StSt, red). Results indicate that the J and E genomic probes hybridise to the same areas of the *Th. elongatum* chromosomes. No blocking DNA was used. Counter-stained with DAPI. The areas labelled with a U represent areas of the chromsomes that remained 'Unbound'.

The near identical results shown in Figure 5.11 indicate the high homology of the J and E genomes. However, in both images, there are regions of chromosomes and whole chromosomes left un-probed as labelled in the figure. This could indicate the possible presence of other unknown genomes in *Th. elongatum* 401007.

To further investigate, two additional slides containing metaphase spreads from *Th. elongatum* 401007 were probed with genomes from other potential progenitor species. The first slide was probed with *Ae. speltoides* (genome = BB)*, Th. bessarabicum* (genome = JJ) and *Ps. stipofolia* (genome = StSt)*.*The second slide was probed with *Secale cereal*e (genome = RR), *T. urartu* (genome = AA) and *Ae. tauschii* (genome = DD).

Figure 5.12 shows the results from the first slide probed with the B, J and St genomes. It revealed that the St, J and B genomes all bound with regions of the *Th. elongatum* 401007 genome. Two images were required to differentiate between the locations the J (gold) and B (green) probes were hybridising too. The regions of the chromosomes binding with the B genome are within the regions that the J genome also binds. Therefore it is unlikely that the B genome is actually contributing to the genomic constitution of *Th. elongatum* 401007. It could suggest that the B and J genome are more closely related than previously thought, or that the J genome contains highly homologous sequences to those found in the B genome.

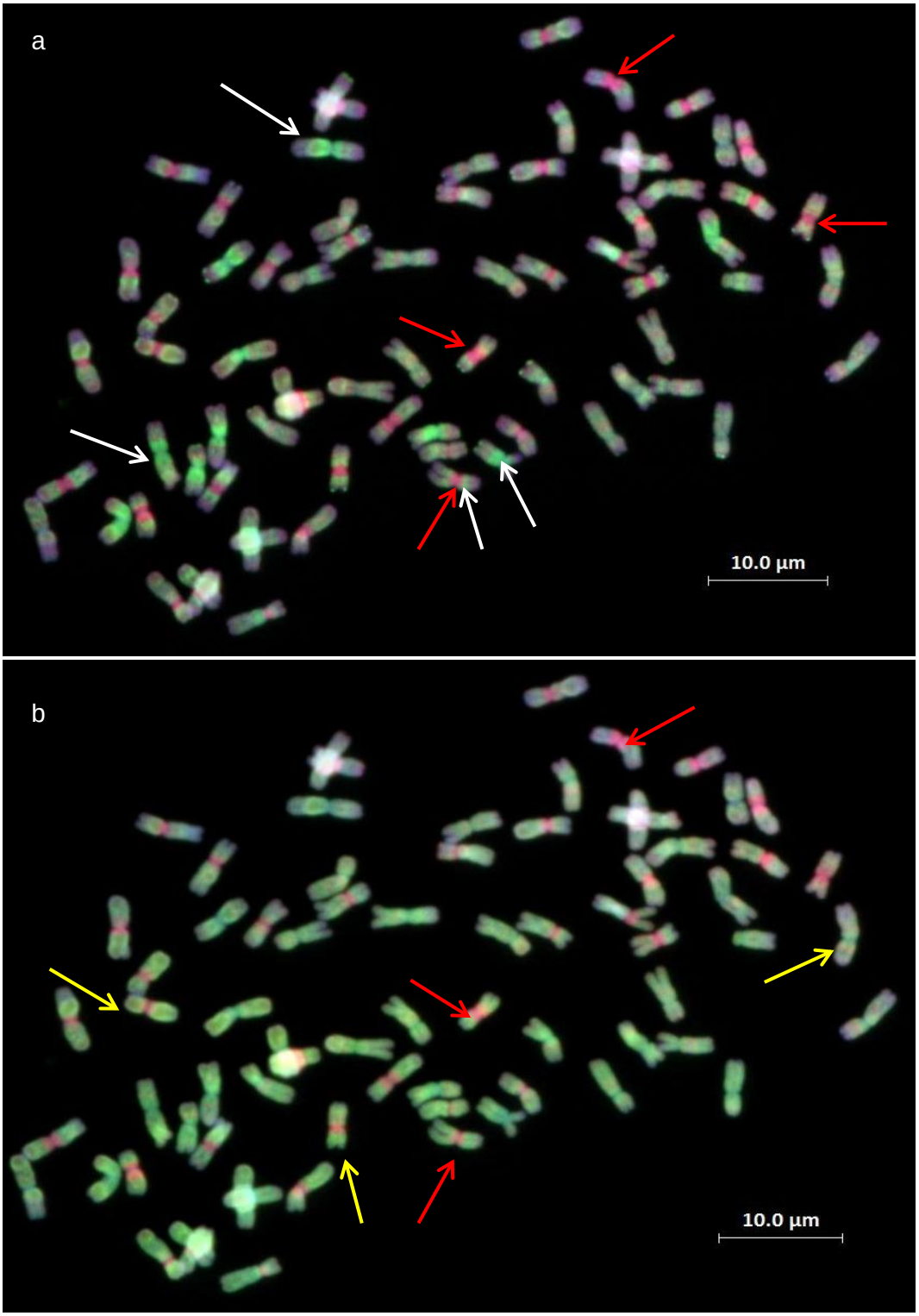


Figure 5.12: Two images of the same metaphase spread of *Th. elongatum* 401007 probed with fluorescently labelled DNA from *Ps. stipofolia*, genome = StSt (red) and *Ae. speltoides*, genome = BB (green) and *Th. bessarabicum*, genome = JJ (gold). (a) shows the St and B (red and green) genomes clearly hybridising to sections of individual chromosomes. Image (b) shows the hybridisation of the J and St genomes (gold and red). Examples of B genome hybridisation is indicated using white arrows, St genome hybridisation is indicated using red arrows and J genome hybridisation is indicated using yellow arrows.

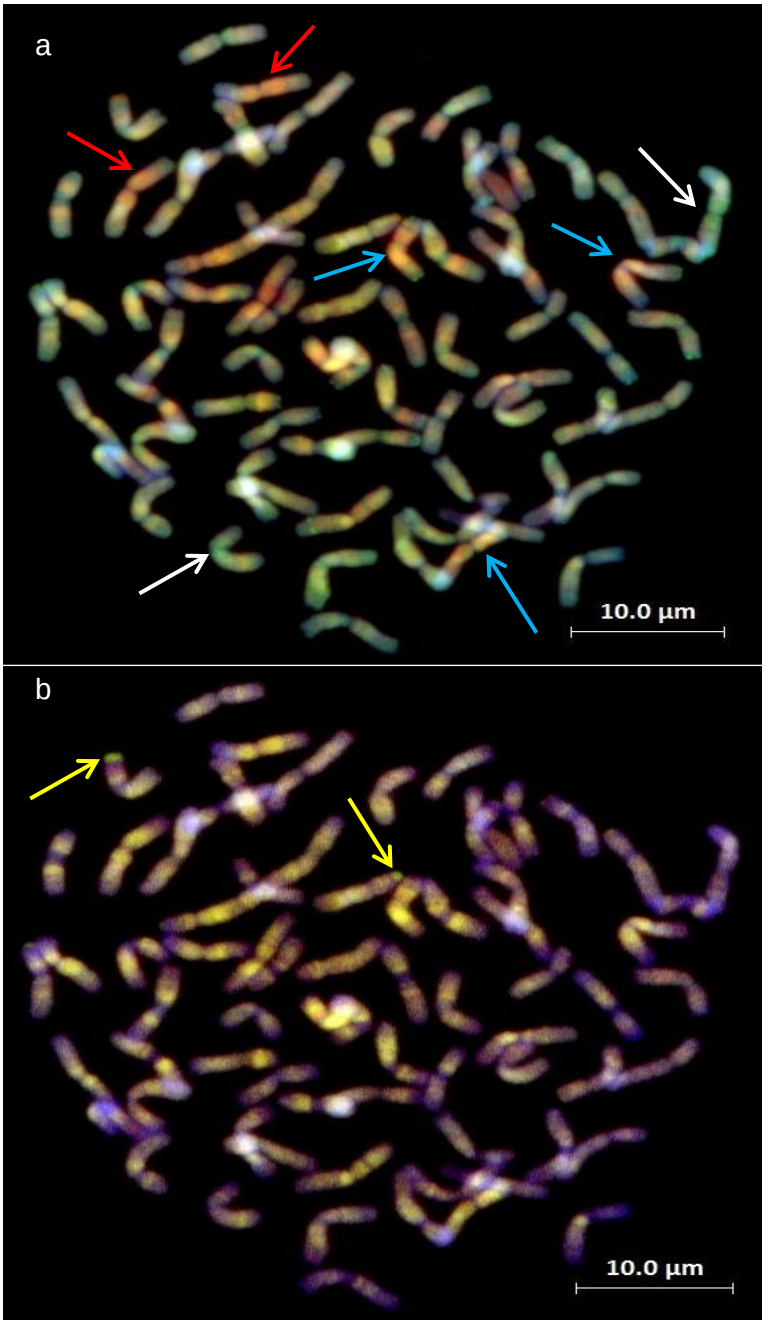


Figure 5.13: Two images of the same metaphase spread of *Th. elongatum* 401007 probed with fluorescently labelled DNA from *T. urartu*, genome = AA (green), *Ae. tauschii*, genome = DD (red) and *S. cereale*, genome = RR (gold). Both images are counter-stained with DAPI. (a) shows the A and D probes hybridising to segments of the *Th.* elongatum chromosomes. The A probe (green) is indicated using white arrows and the D genome (red) is indicated using red arrows. Orange regions of chromosomes from where the A and D genomes appear to have hybridised together are indicated using blue arrows. (b) shows the hybridisation of the R probe. Due to no blocking DNA being used the spread appears to hybridise with the R probe, however the only true hybridisation signals are (indicated using yellow arrows) are at a few telemetric regions. Therefore the R genome is not present in the genome of *Th. elongatum* 401007.

Figure 5.13 shows the results from the second slide probed with the R, A and D genomes. It suggests that some areas of the *Th. elongatum* 401007 genome bind equally with the A and D genomes producing the orange signal seen in part (a) of the figure (blue arrows). Once again the A and D genomes are binding to similar regions that the J genome bound to in Figure 5.12. This suggests that the J genome is also closely related to the A and D genomes of wheat, or that the genomes share conserved sequences. The R probe failed to hybridise except at highly localised telemetric regions indicating the presence of a conserved sequence but no *S. cereale* genome is present in the *Th. elongatum* genome. Future work should be undertaken to probe slides from *Th. elongatum* 401007 with the A, D and J genomes to see if they bind to similar sites.

To summarise, strong hybridisation signals were seen when investigating the *Th. elongatum* 401007 genome from the following genomic probes: B, E, J and St. Weak signals were seen from the A and D genomic probes. No signal was seen with the R and V genomic probes. Areas of the chromosomes remained unstained in all mc-GISH analysis of *Th. elongatum* 401007 indicating the possible presence of at least one other currently unidentified genome.

Due to the complicated nature of the parental decaploid genome, it was decided to investigate some of the introgression lines using a mc-GISH approach with probes made from the total genomic DNA of *Th. bessarabicum* (genome = JJ) and *Ps. stipofolia* (genome = StSt). The J genomic probe was used rather than the E genomic probe due to the results obtained when probing the decaploid *Th. elongatum* 401007 parental (see Figure 5.11) and the increased availability of J total genomic DNA at the time of the experiment.

5.4.2.2. Mc-GISH using the J and St genomes

Thirty-two lines with good quality metaphase slides available and pre-existing sc-GISH results were randomly selected and probed. Of the 32 lines probed, 28 were successful and all 28 matched their corresponding sc-GISH results. From these 28, 14 lines contained one introgression each and 14 lines contained varying numbers of whole chromosomes transferred from *Th. elongatum*. All 14 introgressions were visualised using mc-GISH. Eleven were

J genome introgressions and 3 were J^{St} introgressions. Sixty-four whole chromosomes were visualised, 36 were J chromosomes and 28 were J^{St} chromosomes. Figure 5.14 shows the sc-GISH and mc-GISH of line BC_4-239D. Both images show 42 chromosomes, with one whole and a recombinant chromosome. The mc-GISH shows the introgression is from the J (E) genome of *Th. elongatum* and the whole chromosome is a J^{St} (E^{St}) chromosome.

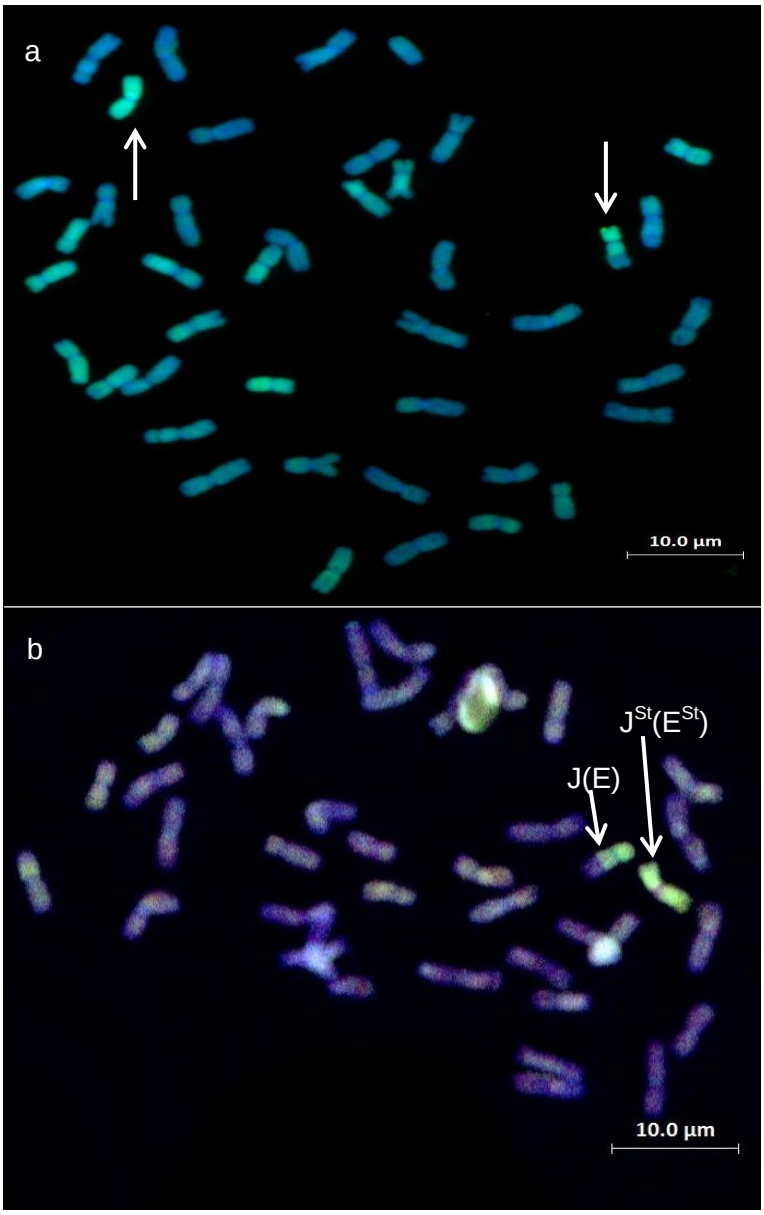


Figure 5.14: Chromosomal spreads from line BC_4-239D probed using (a) fluorescently labelled genomic *Th. elongatum* DNA and (b) fluorescently labelled genomic DNA from *Th. bessarabicum* and *Ps. stipofolia*. Total genomic DNA from Chinese Spring was used as blocking DNA and counter-stained with DAPI. Arrows in (a) indicate a singular introgression and singular whole chromosome. Arrows in (b) show the same introgressions and label the whole chromosome as a modified J^{St} from *Th. elongatum* and the large introgression as a J genome introgression.

Figure 5.15 shows 4 of the 32 lines that underwent mc-GISH using the J and St probes (green and red respectively). The results clearly show that mc-GISH is equally effective at visualising the presence of introgressions and whole chromosomes as well as sc-GISH experiments. Mc-GISH also clearly gives additional information in comparison to sc-GISH, by revealing which genome the introgression has been transferred to in wheat.

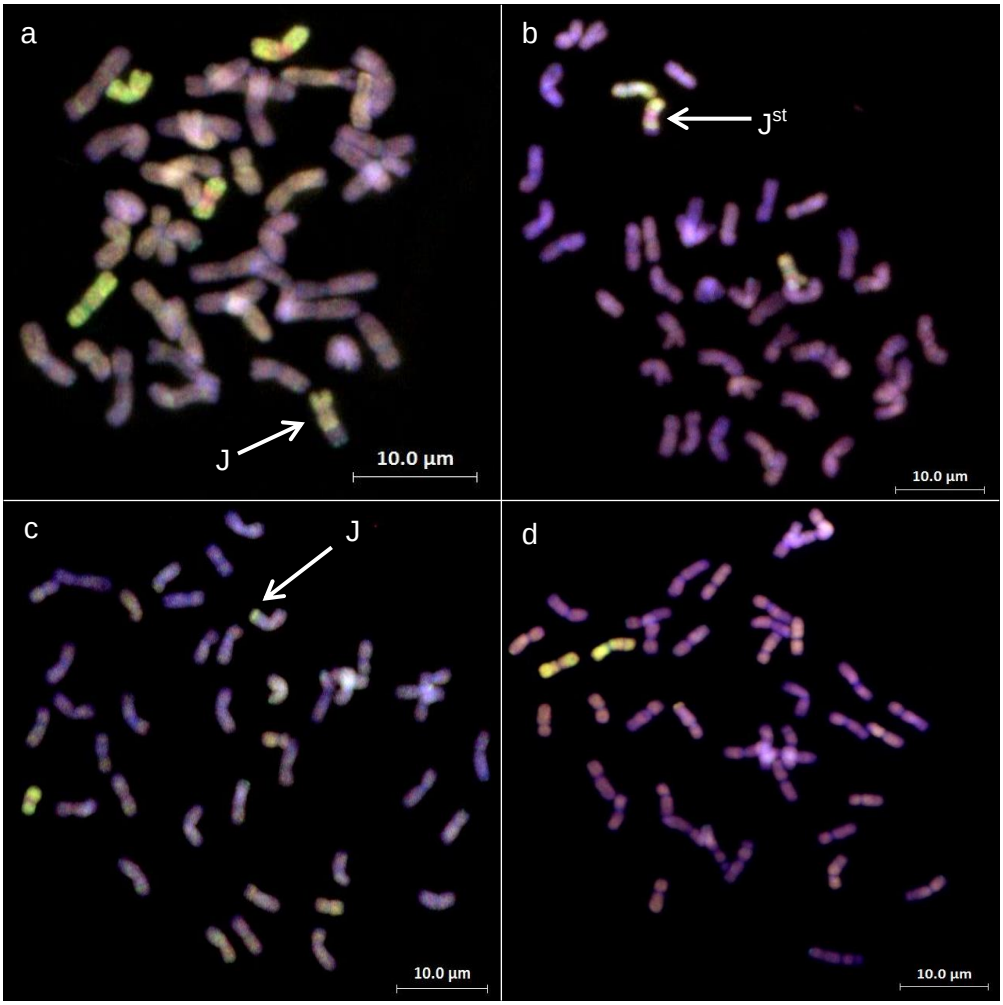


Figure 5.15: Mc-GISH results from four back-crossed lines. All spreads were counterstained with DAPI and Chinese Spring blocking DNA (blue). All introgressions are indicated and labelled using white arrows. All metaphase spreads were stained with fluorescently labelled genomic DNA from *Th. bessarabicum* (green) and *Ps. stipofolia* (red). (a) shows line BC_3-142E with two J chromosomes, 2 J^{St} and a large J introgression. (b) shows BC_3-144E with two J chromosomes and a J^{St} introgression. (c) shows BC_4-179A with one J^{St} chromosome and a small J introgression, and (d) shows BC_4-183A with one J chromosome and one J^{St} chromosome.

5.5. Discussion

5.5.1. Single-colour GISH

The sc-GISH protocol was highly effective at detecting introgression from *Th. elongatum* 401007 in a wheat background, but it was also technically challenging, meaning samples were lost throughout the GISH pipeline. From the final round of breeding, 399 lines underwent root tip digestion (see section 5.3.1) to produce slides with metaphase spreads for subsequent GISH analysis. Variability in root size and toughness limited the success of the digestion of some roots by affecting the effectiveness of the enzyme solution used. Any slides obtained with under-digested root cells were unsuitable for GISH analysis as the probe mixture could not stain the metaphase spreads still within cellular membranes. This resulted in 372 lines being successfully digested to produce metaphase spread slides.

Another technical challenge within the protocol was the probing procedure (see section 5.3.6). Chromosome spreads exposed to temperatures that exceeded the annealing temperature (~75^{o}C) for the probe were prone to melting – resulting in unclear probe binding and sample degradation. This was not visible until final visualisation of the slides on the fluorescent microscope.

However, even with these technical challenges, 372 lines within the breeding programme had their root tips successfully digested and 327 were successfully analysed using sc-GISH. In total, 143 different introgressions were visualised.

The general trend observed throughout the cytogenetics work was that the number of whole chromosomes and introgressions from *Th. elongatum* reduced with each back-cross to wheat. This was not statistically significant and would require a more balanced population to confirm. However, this would be expected as theoretically the wild relative genetic material would be more likely to be selected against during formation of gametes, due to its reduced homology with the wheat genomes.

Even though the number of introgressions and whole chromosomes from *Th. elongatum* decreased in each generation, the numbers seen at later stages of the breeding programme (such as the BC_4 generation) were higher than

expected in comparison to other wild relatives in the same breeding programme (data not published). This could be explained by the decaploid nature of the wild relative used. BC_1s generated from this breeding programme varied in total chromosome number and had a large number of whole chromosomes from *Th. elongatum*. For example, BC_1-454B was shown to have at least 54 chromosomes in total, with at least 12 of those being from the *Th. elongatum* genome. It would therefore be unsurprising for any BC_2 lines generated from this BC_1 to have high numbers of *Th. elongatum* chromosomes remaining.

The maintenance of a large number of *Th. elongatum* introgressions and whole chromosomes could also be explained by the repeatedly reported high homoeology between the A, B, D and E genomes. Further experiments would be required to investigate either possibility.

Regardless of the potential reasons why, the use of sc-GISH in this work proved that the generation of back-crossed lines using the shotgun introgression approach between *Th. elongatum* and wheat is highly successful in generating introgressions between the two species - making *Th. elongatum* an excellent candidate for increasing the genetic variation in the gene pool of wheat. Sc-GISH as a method has also been shown to be excellent for detecting the presence of introgressions in back-crossed lines. A second advantage comes from the increased through-put in comparison to mc-GISH possible as the lowest number of probes is used on each slide. However, it is limited in the amount of information it can provide regarding the genomes transferred and/or the genome of wheat into which the introgression is occurring. It is also still a technically challenging method and is low-throughput in comparison to molecular marker systems.

5.5.2. Parental genome investigation

The results obtained from investigating the progenitors of *Th. elongatum* 401007 were very interesting. Previous literature has offered up three potential genome constitutions for the decaploid of this species, $E^bE^bE^bE^bE^bE^bE^bE^bE^bE^b$ (Arterburn *et al.*, 2011), $JJJJJJJ^sJ^sJ^sJ^s$ (Chen, Conner, Laroche, *et al.*, 1998) and $E^eE^eE^eE^eE^eE^eE^e$StStStSt (Zhang, Dong and Wang, 1996). However, none of these explain the results obtained in section 5.4.2.1. The genomic constitutions $E^bE^bE^bE^bE^bE^bE^bE^bE^bE^b$ and $E^eE^eE^eE^eE^eE^eE^e$StStStSt can both be considered disproved by Figure 5.11, which clearly shows the

presence of the St genome, meaning it cannot be an autopolyploid. It also clearly shows that no whole St chromosomes are present, meaning at this stage the most likely genomic constitution for *Th. elongatum* 401007 is $JJJJJJJ^{St}J^{St}J^{St}J^{St}$ or $EEEEEEE^{St}E^{St}E^{St}E^{St}$.

The J and E genomes both hybridised with the chromosomes of *Th. elongatum* 401007 equally, suggesting they are very similar. This supports research previously published and highlights where the difficulty in classifying *Thinopyrum* species arose. It is possible that both genomes are present within *Th. elongatum* 401007, or it is possible that one genome is a modified version of the other that is so similar that both probes bind to it. Irrespective, at this point it cannot be stated whether just the E genome, the J genome or E and J genomes are present in *Th. elongatum* 401007. The genomes from *Th. elongatum* and *Th. bessarabicum* are too similar to distinguish between using the total genomic DNA probes from this study; they even bound equally to the diploid *Th. elongatum* accession 531718 with the designated genome EE. Future work done to clarify this issue would be required, such as designing unique probes based on the karyotypes of the J and E genomes that have been recently published (Linc, Sepsi and Molnár-Láng, 2012; Linc *et al.*, 2017).

The St genome from *Pseudorogneria stipofolia* was selected for investigation as a progenitor of *Th. elongatum* 401007 due to its presence in *Th. ponticum* and *Th. intermedium*. As expected, the St probe did not bind to the diploid *Th. elongatum* 531718, but the probe was clearly visible on some centromeric regions of chromosomes in *Th. elongatum* 401007 (see Figure 5.12). There were no whole St chromosomes present in the genome of *Th. elongatum* 401007, unlike in *Th. intermedium* (Mahelka, Kopecký and Paštová, 2011). Another additional finding from the mc-GISH work was that the centromeric regions where the St genome bound appears to vary in size and location between chromosomes, suggesting it is not uniform across the *Th. elongatum* genome (see Figure 5.16).

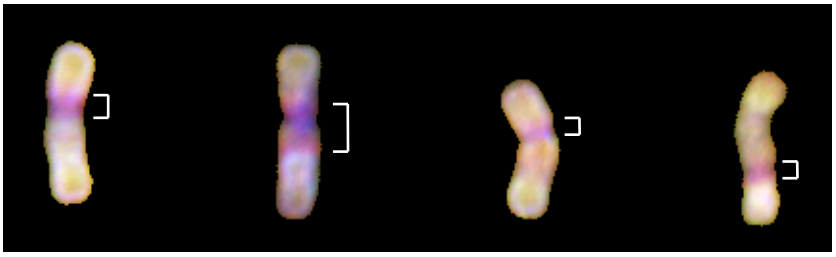


Figure 5.16: Four *Th. elongatum* chromosomes from different individual lines with St genome centromeres of varying sizes and locations. The red regions represent the St genome and are indicated on the figure with the white bars, the light green/yellow areas represent the J genome.

The results from investigating the parental genome did confirm that the V genome is not present in *Th. elongatum* 401007 (see Figure 5.10). This genome was investigated as a possible contributor to *Th. elongatum* 401007 due to its presence in *Th. intermedium* (Cseh 2016, pers. comm.). The R genome from *S. cereale* was also shown not to be present in the genome of *Th. elongatum* 401007. Both probes produced some signals highly localised to telemetric regions suggesting the presence of conserved sequences.

The A, B and D genomes from *T. urartu*, *Ae. speltoides*, and *Ae. tauschii* respectively were then selected to probe metaphase spreads of *Th. elongatum* 401007. The B genome was shown to bind clearly with centromeric regions of *Th. elongatum* chromosomes with and without the presence of the St genome (see Figure 5.12). The A and D genomic probes did not hybridise as strongly as the B genomic probe, and appeared to bind to the same segments of the *Th. elongatum* chromosomes as each other (see Figure 5.13). The combination of the A probe (green) and D probe (red) resulted in an orange signal being produced in varying lengths of centromeric regions. Further experiments using a combination of J or E probes with the A and D probes would be interesting to see which genomes preferentially hybridise along the lengths of the *Th. elongatum* 401007 chromosomes. The A, B and D genomic probes all bound to regions of the *Th. elongatum* 401007 genome that the J genomic probe also bound to. This result suggests that the A, B and D genomes are not actually involved in the genomic constitution of *Th. elongatum* 401007, but are highly syntenic with the J genome. The B genome bound most strongly out of the A, B and D, suggesting that the B genome is most closely related to the J genome, followed by the A and D genomes.

In each mc-GISH experiment, different segments of the *Th. elongatum* segments did not bind with the probes applied, suggesting there may be at least one unidentified genome present. Future work undertaken should involve probes from other genomes of other diploid wild relatives of wheat to see if it's possible to identify all genomes involved in the *Th. elongatum* genome. Different concentrations of the J, E and St probes already shown to hybridise to the *Th. elongatum* 401007 chromosomes, should also be investigated.

Each mc-GISH experiment was limited by the amount of probes that could be visualised at one time. Metaphase slides containing *Th. elongatum* 401007 chromatin probed with three different genomic probes and counter-stained with DAPI (4 colour GISH) could not be visualised in one image. This was not true for all the wild relatives of wheat used in the WRC breeding programme. Introgression lines produced with wild relatives with genomes that are more distinct to the A, B and D genomes of wheat (such as *S. cereale* and *Am. muticum*) could be visualised in one image (King *et al.*, 2017; Heath, 2018). The limit for visualising four probes at once with *Th. elongatum* hybrid lines came from the close synteny between the genomes being investigated resulting in probes binding less specifically. In particular, the *Th. elongatum* 401007 probe (green) and any gold probe were exceptionally difficult to differentiate on a single image, due to the filter from the microscope making them both appear as green. Hence, slides probed with four different probes require two images to view all of the results most accurately.

Future work on this particular accession of *Th. elongatum* should include mc-GISH analysis of *Th. ponticum* to identify if *Th. elongatum* 401007 is possibly a mis-labelled *Th. ponticum*, or to see if *Th. elongatum* 401007 has a unique genomic constitution previously undescribed. Other future studies should investigate other polyploid accessions of *Th. elongatum* to see if they are genomically distinct from their diploid counterparts. The fact that *Th. elongatum* 401007 has a total chromosome number of 72 made obtaining high quality metaphase spread slides very difficult. The other main limitation of this work was the time required to stain and visualise slides with mc-GISH, for each probe added to the mixture another filter was required on the microscope, increasing the image capture time.

5.5.3. Multi-Colour GISH of A, B, D genomes

Mc-GISH can be considered a more powerful tool for investigating introgressions. It revealed intergenomic introgressions between the genomes of wheat as well as between *Th. elongatum* and wheat. For example, BC_5-83A was shown to have one whole chromosome and one small introgression from the sc-GISH results. The mc-GISH results revealed that that introgression actually was a double recombinant, as it has a telemetric introgression between the D and A genomes of wheat as well as the introgression between *Th. elongatum* and the A genome of wheat (see Figure 5.17).

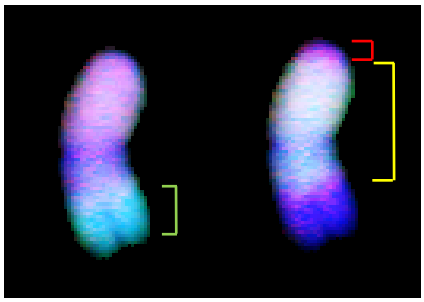


Figure 5.17: The two images are of the same chromosome displaying the same introgression from BC_5-83A. The left hand image shows the bottom green telomere of the A genome from wheat. The right hand image shows the same green telomere has now become blue as the filter on the microscope required changing in order to visualise the green/gold centromeric region from *Th. elongatum* 401007. The right hand image also clearly shows the top telomere of the chromosome is comprised of the D genome of wheat (red). Therefore this introgression is a double recombinant; an E with A and D telomeres.

This mc-GISH experiment aimed to investigate whether *Th. elongatum* introgressions produced in back-crossed lines were preferentially recombining with the A, B or D genome of wheat. Research has repeatedly indicated that the E and J genome are most closely related to the D genome of wheat, then the A and most distantly related to the B genome (Liu, Li and Zhang, 2007; Hu *et al.*, 2012). If the D genome is the most homologous to *Th. elongatum* it is unsurprisingly that a large number of previously reported translocations or substitutions from the *Thinopyrum* genus appear to take place in the D genome of wheat (Friebe *et al.*, 1996; Han *et al.*, 2003; Qi *et al.*, 2007; He *et al.*, 2009; Zheng *et al.*, 2009; Li *et al.*, 2017). Therefore, it was hypothesised that the majority of introgressions produced from this breeding programme would be with the D genome of wheat.

Twenty-nine lines were analysed from a range of generations in the breeding programme (BC_3 to BC_5F_1). From these 29 lines, 32 introgressions were visualised. Lines were selected based on the quality of their sc-GISH results and the availability of high quality metaphase slides. It was expected that, due to the relationship between the genomes, more of the introgressions from *Th. elongatum* would have combined with the D genome in wheat. However, 13 (40.6%) of the introgressions were found to be between *Th. elongatum* and the A genome of wheat, 11 (34.4%) between *Th. elongatum* and the B genome of wheat and just 4 (12.5%) between *Th. elongatum* and the D genome of wheat. The other 4 (12.5%) of introgressions were actually double recombinants involving multiple genomic combinations (see Table 5.3). These results suggest a closer than previously reported genomic relationship between the B and E/J genomes. This finding is supported by the presence of the B genomic probe along regions of the E/J chromosomes in the *Th. elongatum* 401007 genome (see Figure 5.12).

These results were the opposite of what was expected. However, the presence of sequences from the A, B and D genomes of wheat shown in Figure 5.12 and Figure 5.13 could account for why introgressions from *Th. elongatum* are recombining with all 3 genomes of wheat more frequently than previously reported in the literature. It is possible that due to the smaller sample size tested using mc-GISH that these results are not representative of the population. However a larger sample size was not possible due to time constraints and limited germplasm materials. Future work should be focussed on mc-GISH to analyse introgression lines in order to detect any intergenomic introgressions between the wheat genomes at the same time as characterising the introgressions between *Th. elongatum* and wheat. Taking all of this into account, the results of these experiments suggest that *Th. elongatum* 401007 introgressions appear to be most likely to recombine with the A and B genomes of wheat and least likely with the D genome of wheat.

5.5.4. Multi-colour GISH of J and St genomes

Mc-GISH experiments investigating whether different genomes of *Th. elongatum* 401007 were preferentially transferred to wheat were conducted at the same time as the investigation into the progenitor species of *Th. elongatum* 401007. Due to previous research published supporting the high similarity between the E and J genomes, the supporting results shown in Figure 5.11 and the increased availability of J total genomic DNA in the lab, this mc-GISH protocol used J total genomic probes rather than E. Probe made from total genomic DNA of *Ps. stipofolia* was also used (genome = StSt).

Twenty-eight lines were successfully probed with the J and St total genomic probes, whilst using total genomic DNA from wheat (acc. Chinese Spring) as blocking DNA. As with the other mc-GISH experiments, the results perfectly matched those from the sc-GISH experiments and additional information regarding the introgressions transferred was obtained. It was expected that the J genome would preferentially form introgressions with wheat as it was reportedly more closely related to the A, B and D genomes of wheat. Results from the 28 lines supported this hypothesis. Out of the 14 introgressions visualised, 11 were J genome introgressions (78.6%) and 3 were J^{St} (21.4%).

It was observed that none of the J^{St} recombinants were formed from recombination events between the wheat genomes and the St genome region of the J^{St} chromosomes. The recombination event always took place within the J genomic regions of the modified chromosomes. These findings supported the theory that the St genome is more distantly related to the A, B and D genomes of wheat.

From the same 28 lines, 64 whole chromosomes were transferred from *Th. elongatum* 401007 into wheat. Out of the 64 chromosomes, 36 were J genome chromosomes and 28 were J^{St} chromosomes, 56.3% and 43.7% respectively. These results suggest that there is little preferential transfer of the J or J^{St} genomes from *Th. elongatum* 401007 to wheat when a whole chromosome is being transferred. This could possibly be explained by the fact there is no recombination event between the genomes, and so the decreased co-linearity of the J^{St} genome is not such a barrier to transfer.

Whilst the cytogenetic work completed in this research has proven to be extremely successful at detecting and characterising introgressions from *Th. elongatum* 401007 in a wheat background, it has been limited by technical difficulties and is a relatively low-throughput technique. A high-throughput methodology for detecting introgressions using molecular markers would dramatically increase the number of lines that can be screened. However, when dealing with a plant with such a complicated genome as a parental line, finding reliable and robust markers could prove problematic. A combination approach using cytogenetic techniques such as sc-GISH and mc-GISH will help to validate any molecular marker work done. Chapter 6 will attempt to identify molecular markers that can be used to characterise introgressions proven to exist in this chapter. The results from the sc-GISH and mc-GISH will be used to validate marker data obtained.

6. Characterising Introgressions using Molecular Markers and Sequencing

6.1. Introduction

6.1.1. Previous successes using molecular markers to characterise *Thinopyrum* species

The microscope-based, cytogenetic techniques of GISH, FISH and mc-GISH are very powerful tools for identifying introgressions from wild relative species. However, when wanting to screen large numbers of plants, such as in high-throughput breeding programmes, the cytogenetic techniques become exceptionally time-consuming, technically demanding and expensive. Molecular markers are currently expensive to develop, however they would enable a high-throughput approach to be undertaken. Whilst the availability of markers for *Thinopyrum* species is limited, a combinational approach of cytogenetic methods and molecular methods would continually increase the speed and accuracy at which introgressions in a wheat background can be detected and characterised.

There have been several notable successes of transferring genetic variation from *Thinopyrum* species into wheat, using a variety of markers to identify successfully transferred segments. Firstly, morphological markers have been frequently used when transferring genes from *Thinopyrum* species associated with disease and pest resistance, as it is simple to test for the trait by exposing the lines to the relevant disease or pest (Li *et al.*, 2018, Turner *et al*. 2013, etc). Another morphological marker of interest in the *Thinopyrum* family is the blue-grained gene (*Ba* gene). The presence of the *Ba* gene has been reported and studied in the polyploids, most notably in *Th. intermedium* and *Th. ponticum*. It is known that the blue grain phenotype is caused by the deposition of anthocyanin compounds in the aluerone layer of seedlings due to an upregulation of the flavonoid biosynthesis pathway (Zheng *et al.*, 2009). Due to its clear distinguishable phenotype, the *Ba* gene has been used as a screenable marker in genetic and breeding studies of wheat. For example, Hanson *et al.* (2005) used winter wheat with the *Ba* gene to investigate distance of outcrossing between wheat cultivars.

With advances in current marker technology, the importance of morphological markers such as the blue-grained gene is questionable in future research. Morphological markers in general are limited in numbers and can often only be visualised in adult phenotypes. In high-throughput breeding programmes this can result in a large waste of resources just getting the plant material to maturity before useful lines can be identified. These markers would have been increasingly problematic in the current programme due to the wheat/*Th. elongatum* back-crossed lines reaching maturity very slowly. Morphological markers can also be dosage dependent, which again would be increasingly complicated when working with lines produced between hexaploid wheat and decaploid *Th. elongatum*. In addition to these limitations, studies using morphological markers to identify lines of interest are usually only based on one or two traits of interest, resulting in the majority of the variation available in the *Th. elongatum* genome being ignored.

Molecular markers should enable the entirety of the variation present in *Th. elongatum* 401007 to be harnessed. As discussed in section 1.7.1, ideal properties of DNA markers should include a high abundance and high polymorphic nature, a low mutation rate and a selectively neutral behaviour. They should also be easy to isolate, reproducible, cheap and be suitable for automation to be advantageous for high-throughput studies.

There have been many previous successes using gel based markers such as RFLPs (King *et al.*, 1997; Han *et al.*, 2003), RAPDs (Liu *et al.*, 1998) and AFLPs (Prins *et al.*, 2001) to identify *Thinopyrum* genomic material in back-crossed lines. More recently RAPD primers have been used to develop SCAR (sequence characterised amplified region) markers that are genome and chromosome specific for *Th. elongatum* (Xu *et al.*, 2012; Gong *et al.*, 2013). However, the major downside of the gel-based assays is that they actually require gels to visualise the products, making them time-consuming, laborious and low-throughput. DNA sequencing markers were developed to decrease the time and cost of molecular work.

Previous studies revealed that the majority of molecular work completed with *Th. elongatum* have used diploid accessions. The fact that diploid *Th. elongatum* has undergone more studies than the polyploid accessions is most likely due to the simplicity of the diploid genome and the availability of well-defined cytogenetic materials including multiple addition, substitution, translocation and ditelosomic lines (Dvorak and Knott, 1997; Lou *et al.*, 2017).

Molecular markers have also been used to show genomic relationships between wheat and *Thinopyrum* genomes. As previously discussed, the E genome from diploid *Th. elongatum* has been shown repeatedly to be most closely related to the D genome of wheat (Liu, Li and Zhang, 2007; Hu *et al.*, 2012). This synteny between wheat and the *Thinopyrum* species has been shown using genomic-SSRs (G-SSRs) and express sequence tag SSRs (E-SSRs) (Zhang *et al.*, 2011). The same study used these markers to create a genetic map of chromosome 7E from *L. ponticum* (*Th. ponticum*) and mapped the major FHB resistance locus (*FbhLoP*) to the distal region of the chromosome whilst identifying flanking markers. Whilst SSR markers in general are very powerful, abundant and show high levels of polymorphism they are susceptible to giving null calls if a mutation occurs within their primer site. The likelihood of null calls being incorrectly detected increases with ploidy, due to polymorphic SNPs having the potential to be present in different dosages. The development of such markers also proves challenging and expensive if primer sequences for the species of interest are unavailable (Kesawat and Das, 2009). Considering that the genomic and molecular markers sequences for *Thinopyrum* species, especially polyploids, are still limited these markers are also limited in their effectiveness (Mahelka, Kopecký and Paštová, 2011). The development of more markers between the *Thinopyrum* species would be beneficial for improving molecular mapping, detecting genomic transfer into wheat and for resolving the phylogenetic relationships of the genomes in the *Thinopyrum* genus (see section 4.1.1).

Molecular markers can also be used to investigate the phylogenetic relationships between species. The phylogenetic relationships between *Thinopyrum* species, *Triticum* species and *Hordeum* were investigated using SCoT (Start Codon Targeted) and CDDP (Conserved DNA-Derived Polymorphism) markers (Guo *et al.*, 2016). SCoT and CDDP markers are in principle similar to RAPD markers, however SCoT markers differ because they are based on the short conserved region that is either side of the ATG initiation codon (Collard & Mackill 2009a) and CDDP markers differ as they are designed to exploit conserved DNA polymorphisms in genes known to play a role in both abiotic and biotic stress tolerances (Collard & Mackill 2009b). RAPD markers, and therefore SCoT and CPPD markers, are advantageous in that they are technically simple and no previous sequence knowledge is required. The results from the study are shown in Figure 6.1. To

accurately discriminate between accessions of *Th. elongatum*, especially between accessions with different ploidy, more powerful, genome-specific markers will need to be developed and evaluated.

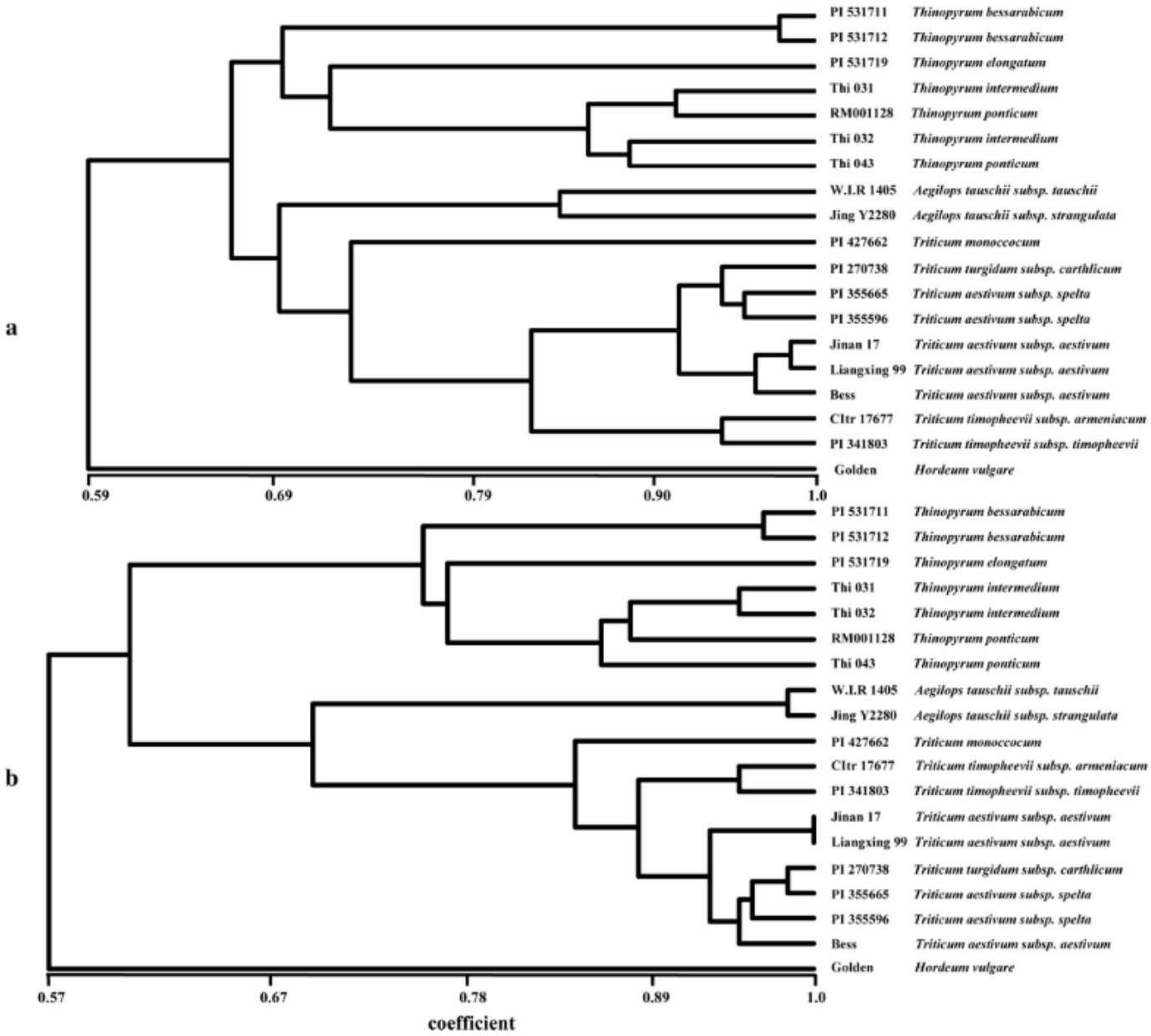


Figure 6.1: Genetic relationships among *Thinopyrum*, *Triticum* and *H. vulgare* constructed by the un-weighted pair-group method for (a) 17 SCoT markers and (b) 10 CDDP markers. The scale indicates the coefficient. These dendograms are taken from Guo *et al* (2016).

Harnessing the depth and breadth of genetic variation available in the *Thinopyrum* genus, as well as other wild relatives of wheat, has been hindered by the lack of a high-throughput sequencing technology to identify and characterise introgressions lines rapidly in a back-crossing programme. The lack of reference sequence libraries for *Thinopyrum* species and minimal marker development, in particular for polyploid accessions, has also limited progress. The first draft of the hexaploid wheat genome was only released in 2014 (IWGSC, 2014). The development of next-generation sequencing, genotyping-by-sequencing and advances with SNP markers over the last 18 months, combined with the decreasing costs of sequencing samples is enabling the identification of introgressions to become increasingly rapid and cheap (see section 1.7.1 for a more detailed introduction to molecular markers). It has also dramatically increased the number, and quality, of reference sequences (including those for wheat) and markers available for comparative analysis of wheat/*Thinopyrum* introgression lines (Xu *et al*. 2012; Gong *et al*. 2013; IWGSC, 2014; IWGSC, 2018). Consensus sequence maps are starting to be released for some *Thinopyrum* species by observing collinearity and synteny between barley and wheat reference genomes (Kantarski *et al.*, 2017). In spite of these advances, markers used for *Th. elongatum* 401007 must be carefully selected, as most previous marker studies have been conducted with diploid accessions of *Th. elongatum*, and therefore the markers from such studies are most suitable for detecting the E genome and less suited for the multiple genomes in *Th. elongatum* 401007.

A recent development in *Th. elongatum* molecular markers was the 35,193 SNP markers which were recently mined for the E genome using diploid *Th. elongatum* (Lou *et al.*, 2017). Markers were validated using a Chinese spring/*Th. elongatum* octaploid and 89% were shown to be authentic. Such high-through put SNP mining will dramatically aid future genetic studies done with germplasm created between wheat and *Th. elongatum* in the future.

Another recent success for molecular markers in the *Thinopyrum* genus comes from research published by the Nottingham BBSRC WRC. The research used 1,150 SNP markers it developed to create a putative linkage map for the genome of *Th. bessarabicum.* This map enabled comparative analysis showing conserved synteny between the genomes of wheat and *Th. bessarabicum*. It also showed that the 4/5 translocation in the A genome of wheat is present in *Th. bessarabicum* (Grewal *et al.*, 2018).

6.1.2. Using molecular markers to characterise WRC wheat/*Thinopyrum* introgression lines

The Nottingham BBSRC Wheat Research Centre (WRC) is currently developing genome-wide introgression lines produced via a whole genome shotgun approach between wheat and ten wild relatives of wheat. The wild relatives included in the programme are: *Am. muticum, Ae. speltoides, Ae. caudata, T. timopheevii, T. urartu, S. cereale, Th. bessarabicum, Th. elongatum, Th. intermedium* and *Th. ponticum* (King *et al.*, 2017). In order to detect any introgressions or chromosome segments from these wild relatives in a wheat background a 35K Axiom® SNP array has been developed (Affymetrix, (now Thermo Fisher Scientific), California). Each SNP included in this array shows polymorphisms between wheat and one or more of the ten wild relatives in the study. The SNPs that form the 35K Axiom® Wheat Relative SNP array are part of the Axiom® 820K SNP array (Winfield *et al.*, 2015). The data set for the Axiom® 820K array is available from www.cerealsdb.uk.net (Wilkinson *et al.*, 2012). It was first available on CerealsDB 2.0, however CerealsDB 3.0 is the latest version of the online resource designed to assist breeders and scientists select the most useful markers for marker-assisted selection (Wilkinson *et al.*, 2016).

Collaborators at Bristol University completed exome capture on all ten of the wild relatives in the University of Nottingham breeding programme, including *Th. elongatum* 401007. Exome capture was also completed on the landraces, elite cultivars and the four accessions of wheat used in the breeding programme: Paragon, Chinese Spring, Highbury and Pavon 76. The aim of this exome capture, with regards to the WRC, was to identify as many SNPs between wheat its 10 wild relatives (Winfield *et al* 2012).

As many of the SNPs obtained through the exome capture as possible were converted into an array based platform (the 820k array). However, due to its sheer size and the inclusion of landraces, elite cultivars, wild relatives and wheat accessions, this array is very expensive to buy and run. Therefore, subset arrays were made, most notably the Breeders Array and the Axiom® Wheat-Relative Genotyping Array. Both of these arrays are 35K SNP arrays, the first was designed to reveal SNPs between accessions of wheat,

landraces and elite cultivars, the second was designed to reveal SNPs between wheat and wild relatives of wheat.

After successfully using the Axiom® Wheat-Relative Genotyping Array to do the initial genotyping, it was decided that a more flexible and cost-effective system was required for genotyping the latter generations of the breeding programme. Thus, Kompetitive allele specific PCR (KASP™) markers were designed to increase the flexibility, speed and cost per sample in comparison to the Affymetrix SNP markers. The Affymetrix plate had to be run with 384 samples and tested each sample against each probe on the array. The turn-around time for this analysis was 6-8 weeks. In comparison, the KASP™ markers can be run with variable number of samples, and variable number of assays as required. The KASP™ markers were suitable for the in-house genotyping platform, dramatically reducing the turn-around time. The KASP™ markers also enable bi-allelic scoring of SNPs, and if genome specific for the A, B and D genomes of wheat, would enable rapid distinction between heterozygous and homozygous introgressions and the identification of the wheat genome involved in the recombination event. The very first KASP™ markers were designed by KBioscience for in-house genotyping and are now also available from LGC Genomics (http://www.lgcgenomics.com). KASP™ markers form a highly affordable uniplex SNP genotyping platform with applications for QTL mapping, allele mining and marker-assisted breeding. However, the use of KASP™ markers for detecting introgressions from *Th. elongatum* 401007 in a wheat background is novel. An excellent review on the function and application of KASP™ marker technology was published recently (Semagn *et al.*, 2014).

6.2. Aims and Objectives

The main aims for this chapter are:

- To detect and characterise introgressions from *Th. elongatum* 401007 in a wheat background using the wheat/wild relative SNP array (Axiom® 35k genotyping array) and Affymetrix SNP markers.
- To detect and differentiate between homozygous and heterozygous introgressions using KASP™ markers.
- To compare introgressions detected via molecular markers to cytogenetic results where possible.
- To produce a genetic map of *Th. elongatum* 401007 using SNP and/or KASP markers produced by this research.

Due to the use of two different marker systems to characterise introgressions between *Th. elongatum* and wheat the remainder of this chapter is divided into two sections. There will be a materials and methods section followed by a results and discussion session for the Affymetrix SNP markers and a separate materials and methods, results and discussion for the KASP™ markers. This division of the work also reflects the timeline that this work was completed in.

6.3. Materials and Methods for Affymetrix SNP markers

6.3.1. SNP marker identification

DNA samples from 352 individual lines produced from the wheat/*Th. elongatum* back-crossing programme were genotyped using the Axiom® Wheat-Relative Genotyping Array and the Affymetrix GeneTitan® system, according to the procedure described by Affymetrix (Axiom® 2.0 Assay Manual Workflow User Guide Rev3). DNA samples were sent to Bristol University in sets of 4 x 96 well PCR plates as the construction of the Array required 384 samples to be run at once.

Allele calling was carried out using the Affymetrix proprietary software packages Affymetrix Power Tools (APT) and SNPolisher™ (http://www.affymetrix.com/estore/partners_programs/programs/developer/tools/devnettools.affx). A custom software pipeline ADAP (Axiom® Data Analysis Pipeline) was written in perl to simplify the data analysis, following the Axiom® Best Practices Genotyping Workflow (http://media.affymetrix.com/support/downloads/manuals/axiom_genotyping_solution_analysis_guide.pdf). A variant call rate threshold of 80% was used instead of the default value (97%) to account for the lower call rates typically obtained from hybridising wheat relatives and progenitors to the array. The APT- probeset-genotype program within Affymetrix Power Tools determines genotype calls from Affymetrix SNP microarrays. Following this, the SNPolisher R package calculates SNP performance metrics, such as call rate, cluster separation and deviation from expected cluster position. It then classifies the SNPs into performance categories. These categories were as follows: (i) 'Poly High Resolution' (PHR), which were codominant and polymorphic, with at least two examples of the minor allele; (ii) 'No Minor Homozygote' (NMH), which were polymorphic and dominant, with two clusters observed; (iii) 'Off-Target Variant' (OTV), which had four clusters, one representing a null allele; (iv) 'Mono High Resolution' (MHR), which were monomorphic; (v) 'Call Rate Below Threshold' (CRBT), where SNP call rate was below threshold but other cluster properties were above thresh- old; and (vi)' Other', where one or more cluster properties were below threshold. Initially, only the PHR SNPs were used as they provide good cluster resolution where each SNP essentially

behaves as a diploid. However, a lack of sufficient markers obtained through the PHR call for *Th. elongatum* 401007 also required the CRBT SNPs to be included in the subsequent analysis.

The above protocol is taken from King *et al* (2017) and was carried out by Affymetrix and Dr. Surbhi Grewal for all ten wild relative breeding pipelines in The Nottingham BBSRC Wheat Research Centre introgression programme.

Once PHR and CRBT SNP markers were identified they were visualised using Flapjack™ (Milne *et al.*, 2010). Any markers that produced heterozygous calls for wheat or *Th. elongatum*, showed no polymorphism between the parents or produced null calls were removed as a quality control (see Figure 6.2).



Figure 6.2: A subset of the PHR/CRBT SNP data after markers showing no polymorphisms between parents, null calls, or heterozygous between the parents have been deleted. The top four rows are the four accessions of wheat and always have a different SNP (shown in either red or green) to the *Th. elongatum* which is shown on row 5. A second sample of the 5 controls is repeated in rows 6-10. The back-crossed line results are in the rows below and show a range of heterozygous, homozygous or null calls.

Remaining markers were then analysed in JoinMap® 4.0 (van Ooijen, 2011). JoinMap® 4.0 sorted the markers into linkage groups using the Haldane map function (Haldane, 1919) to analyse the frequency at which markers appear together, using a LOD score of 20 and a recombination frequency of 0.1. The highest ranking linkage groups with marker numbers higher than 20 were selected, exported and assigned to a chromosome group using chromosome

locations for each marker described in the Axiom® Wheat HD Genotyping Array (Winfield *et al.*, 2015). Once visualised, any markers showing more than 20% erroneous calls were removed as a quality control. Markers at the same genetic position were ordered by their physical positions on the wheat reference genome. This was done by performing a BLAST analysis of the marker sequences against wheat reference sequence (RefSeq v1.0: IWGSC, 2018) and obtaining the best BLAST hit (BBH) from each of the three genomes of wheat, where available. Once complete genotypes for individual lines could be visualised using Graphical GenoTypes 2.0 (Figure 6.4; van Barloo, 2008).

6.4. Results for Affymetrix SNP markers

In total, 263 Affymetrix SNP markers detected 202 introgressions from 352 lines: 26 introgressions in linkage group 1, 24 in linkage group 2, 30 in linkage group 3, 55 in linkage group 4, 26 in linkage group 5, 19 in linkage group 6 and 22 in linkage group 7.

It should be noted that initial Affymetrix SNP marker analysis failed to produce genotypes which matched their corresponding GISH images. This was due to incorrect selection of linkage groups in JoinMap® 4.0. It was originally assumed that there were two genomes present in *Th. elongatum* 401007 based on previous literature and from the initial grouping of markers. Therefore, a minimum of two JoinMap clusters per chromosome (2 for 1E, 2 for 2E etc.) were taken forwards and later forced to combine to form 7 linkage groups. However, the genotypes produced in GGT 2.0 using this assumption did not correspond with the GISH images of the lines. Therefore, this analysis was paused whilst more cytogenetic research was carried out on the genome of *Th. elongatum* 401007 (see section 5.4.2.1). The results from the cytogenetic experiments on *Th. elongatum* 401007 revealed a very complicated genomic structure, which helped explain why the genotyping of the lines was proving so challenging. Once the cytogenetic analysis on the parental lines were concluded, a re-selection of linkage groups and re-ordering of markers was completed based on the assumption the decaploid is essentially behaving as a diploid (E genome) during recombination which would result in 7 linkage groups, not 14. These results matched their corresponding GISH results. The downstream genetic mapping is outlined below to detect the 202 introgressions stated above.

6.4.1. SNP marker selection and the genetic map

In total, 352 lines were analysed using the Axiom® Wheat-Relative Genotyping Array. This number included 17 Chinese Spring/ *Th. elongatum* addition lines, obtained from the John Innes Centre Germplasm Resource Unit (Appendix 9.3) for validation purposes. All lines sent for Affymetrix analysis are shown in Appendix 9.2. In total, 1,594 polymorphic markers between wheat and *Th. elongatum* were selected from the 35K Axiom Wheat-Relative Genotyping Array that were either PHR or CRBT SNP markers with good cluster resolution. These markers then underwent the first quality control check using Flapjack™ and 497 erroneous markers were removed (see Figure 6.2). The remaining 1,097 polymorphic SNP markers between wheat and *Th. elongatum* were then analysed in JoinMap®4.0. The 7 highest ranking linkage groups with more than 20 markers were selected and were assigned chromosomes using BLAST against the wheat reference sequence. The markers for each linkage group then underwent a second visualisation quality control step that removed markers with unique or inconsistent patterns of segregation. This resulted in 263 polymorphic SNP markers used to construct linkage group maps in JoinMap®4.0. Table 6.1 shows the number of SNP markers in each linkage group before the final reordering using JoinMap ®4.0.

Table 6.1: The 263 SNP markers selected from JoinMap® 4.0 to construct individual linkage groups.

Linkage Group	1	2	3	4	5	6	7
Number of SNP markers	68	44	35	32	19	39	26

The cM lengths of the seven linkage groups were 22.08 (1E), 30.83 (2E), 37.88 (3E), 32.83 (4E), 31.74(5E), 15.80 (6E) and 16.67 (7E). These linkage groups were used in the construction of a genetic linkage map using the recombination frequencies of the Affymetrix SNP markers calculated in JoinMap® 4.0 between wheat and *Th. elongatum* 401007, with a total map length of 187.83cM (Figure 6.3).

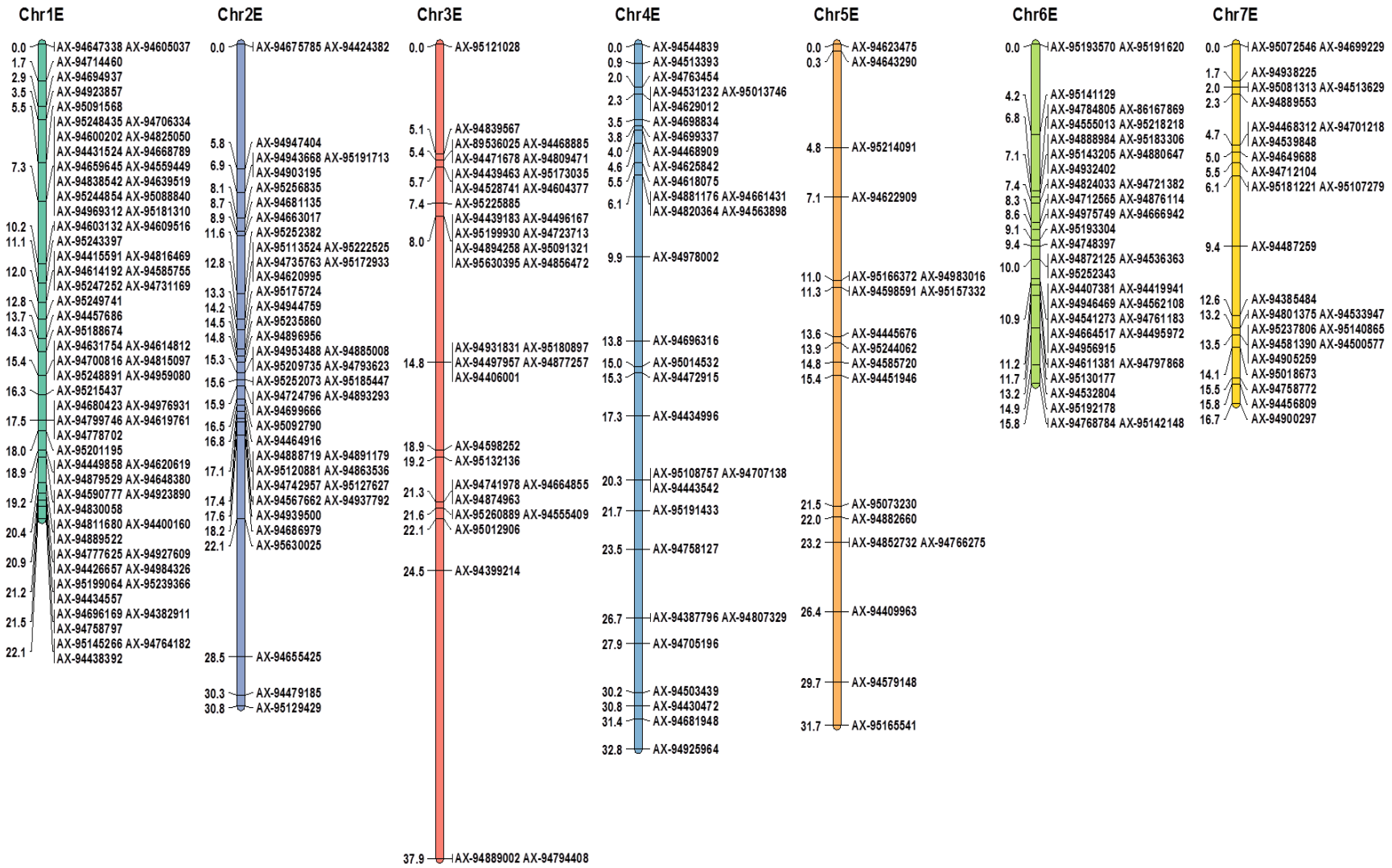


Figure 6.3: The genetic linkage map of the Affymetrix SNP markers for the E genome from *Th. elongatum* 401007. SNP marker names and calculated cM distances for each group is shown.

6.4.2. Marker validation

The GGT genotypes produced using the Affymetrix SNP markers matched their corresponding GISH images. The Chinese Spring addition lines analysed with the Affymetrix markers also matched their known genotypes. Figure 6.4 shows the GGT results for the Chromosome 2 addition line (CS-2E), the Chromosome 2 short arm addition line (CS-2ES) and the Chromosome 2 long arm addition line (CS-2EL). The figure shows the markers are identifying the whole of chromosome 2 in addition line CS-2E, the short arm of chromosome 2E in addition line CS-2ES and the long arm of chromosome 2 in addition line CS-2EL.

Figure 6.5 shows the sc-GISH and corresponding GGT genotype file for line BC_4-89D. The sc-GISH image clearly shows the presence of one whole chromosome and a single introgression from *Th. elongatum* 401007 in a wheat background. The GGT file supports this and identifies the whole chromosome as coming from 1E and the introgression as coming from 5E.

The validation of the markers via cytogenetic techniques supports the use of the linkage map (Figure 6.3) and SNP markers included within to identify and track introgressions between wheat and *Th. elongatum* 401007.

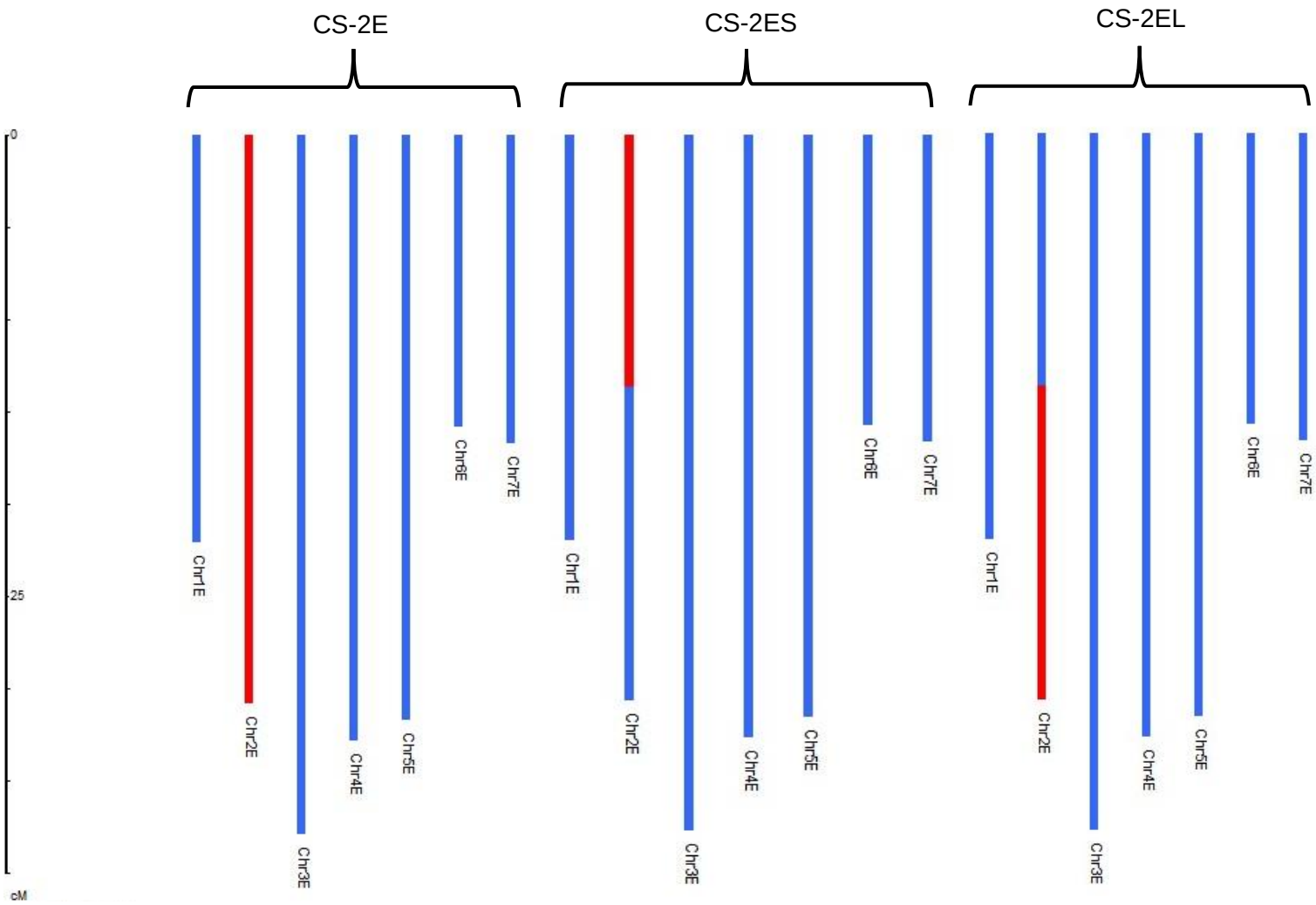


Figure 6.4: The GGT results for the Chinese Spring chromosome 2 addition lines, CS-2E, CS-2ES and CS-2EL. Blue regions represent wheat DNA, red regions represent *Th. elongatum* DNA.

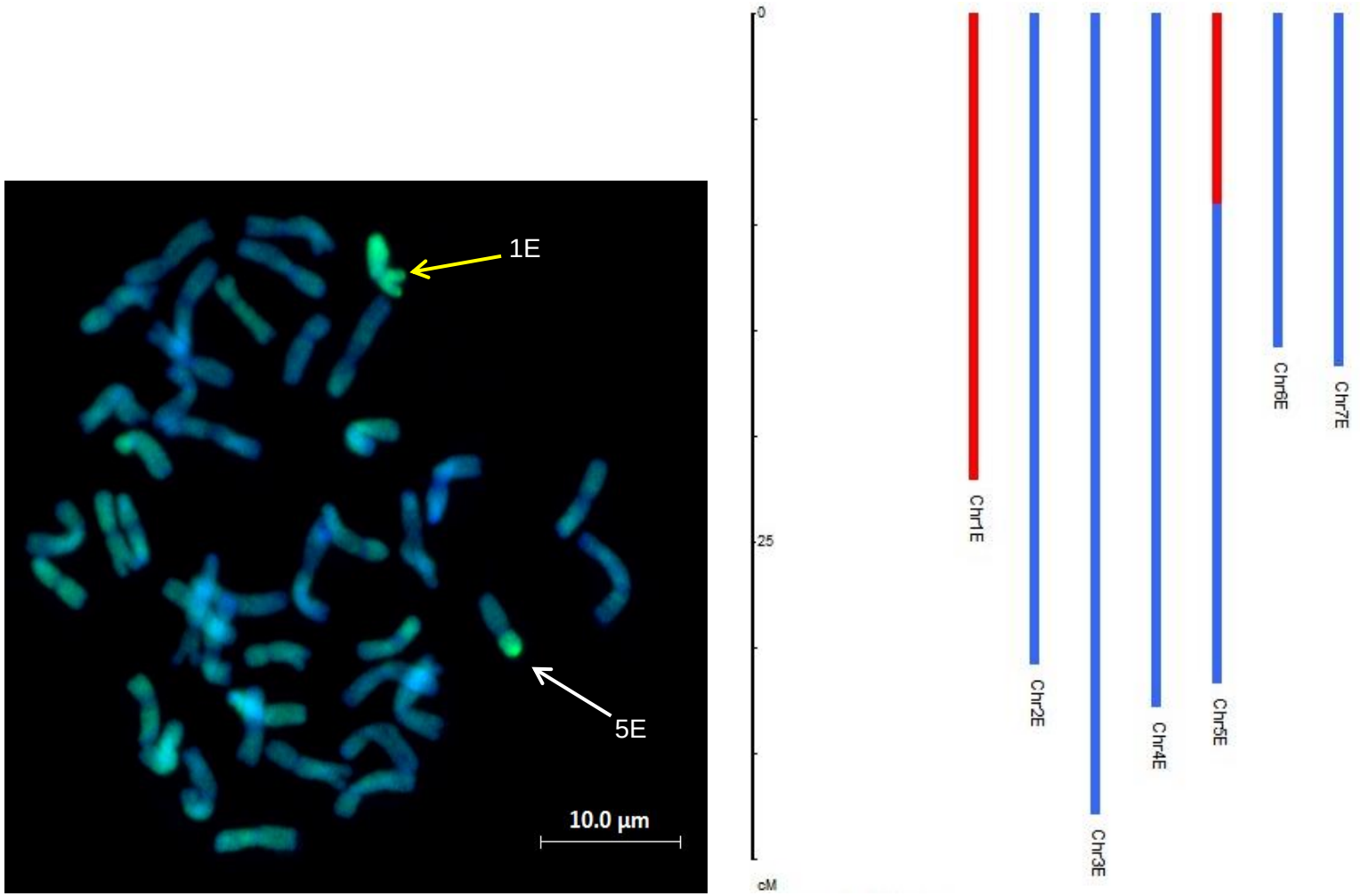


Figure 6.5: The sc-GISH and GGT genotype for line BC_4-89D. The GISH image shows a single whole chromosome (yellow arrow) and a single introgression (white arrow). The GGT genotype corresponds with the GISH image and suggests that the whole chromosome is from 1E and the introgression is from 5E. For the GGT genotype, the red regions indicate *Th. elongatum* DNA and the blue regions represent wheat DNA. The scale bar included on the GGT image shows the distance in cM.

6.4.3. Combining cytogenetic and molecular data

Cytogenetic analysis completed in section 5.4.1 revealed the presence of 143 introgressions from 329 individual plants. However, some questions remained about the introgressions within 'sibling' lines. For example, BC_4-172A, B and C were all shown using sc-GISH to contain a single introgression in a wheat background. The size of the introgression appeared to be the same in each line; however without molecular markers it was difficult to say for sure whether the introgression was actually the same. The validated Affymetrix SNP markers showed that the introgression found in all three lines appears to be the same and is from chromosome 7E of *Th. elongatum* (Figure 6.6).

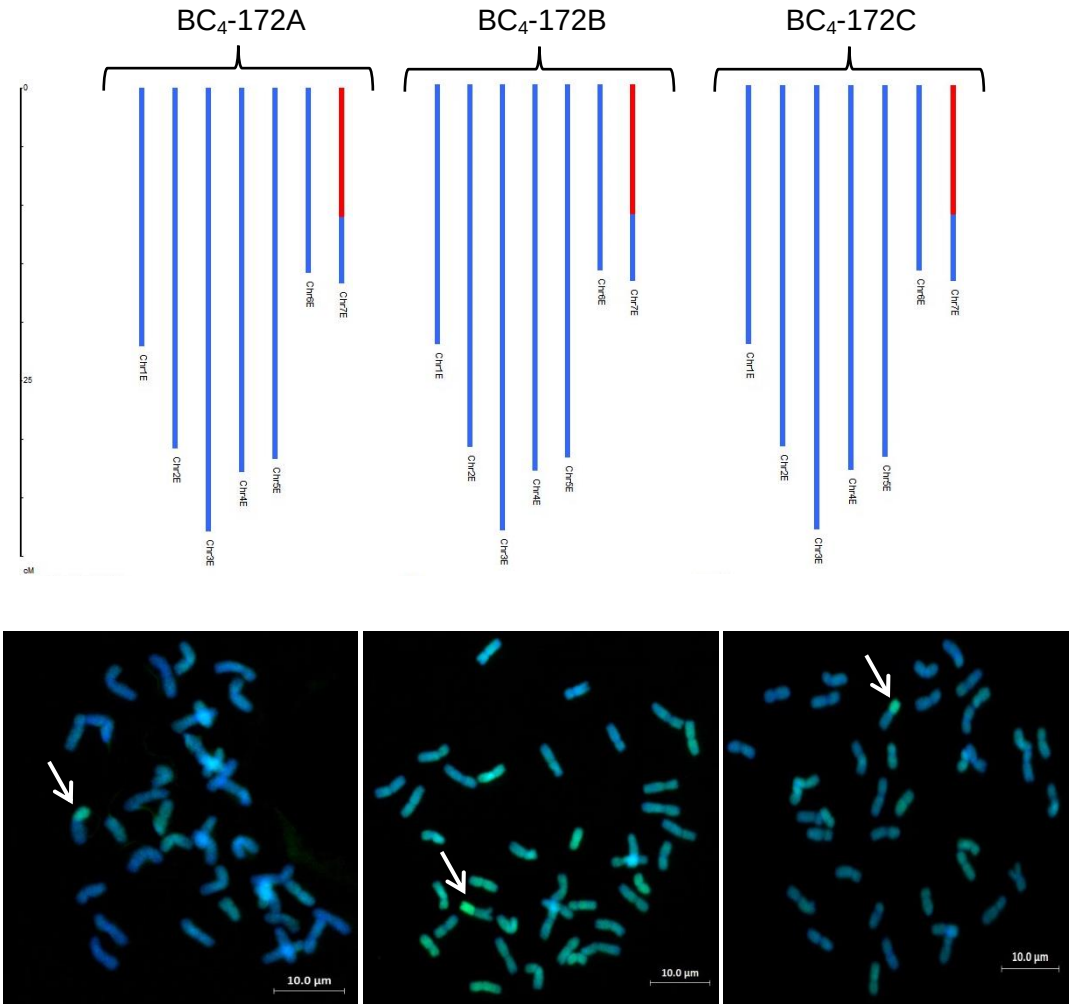


Figure 6.6: The genotype and sc-GISH result for BC_4-172A, BC_4-172B and BC_4-172C respectively. For the GGT files, blue regions represent wheat DNA, red regions represent *Th. elongatum* DNA. The introgression is shown in the GGT genotypes as a red region with chromosome 7E. For the sc-GISH images the introgressions are probed green and indicated with a white arrow.

The molecular markers also identified introgressions within the BC_4-179 family. Sc-GISH results had shown the presence of one whole *Th. elongatum* chromosome and one small telemetric introgression in BC_4-179A, nothing in BC_4-179B, one large introgression and two whole *Th. elongatum* chromosomes in BC_4-179C and a single large introgression in BC_4-179D. Initial analysis of the sc-GISH images suggested the possibility that BC_4-179C and D contained the same large introgression, whilst BC_4-179A could possibly contain the reciprocal introgression from the same recombination event. However, the molecular markers revealed that the small introgression in BC_4-179A was actually from chromosome 4EL whereas the large introgression from BC_4-179C and D was from chromosome 2EL, and therefore the introgressions were not reciprocal, but entirely different (Figure 6.7).

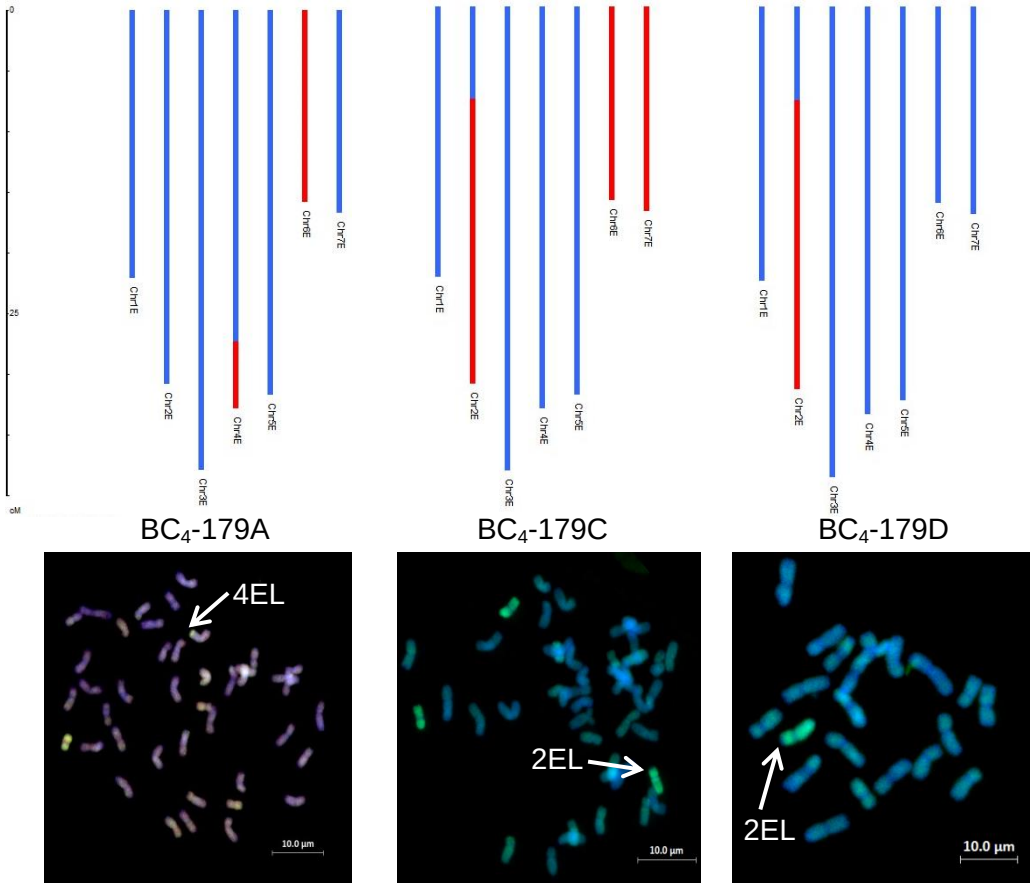


Figure 6.7: The GGT genotype files for BC_4-179A, BC_4-179C and BC_4-179D respectively with their corresponding GISH images beneath them. For the GGT files, red regions represent *Th. elongatum* 401007 and blue regions represent wheat. Within the GISH images, introgressions are labelled using a white arrow.

The molecular markers were also shown to be capable of tracking introgressions through subsequent generations. Figure 6.8 shows the GISH images and genotypes for BC_3-392A and offspring line, BC_4-181B. Both GISH images clearly show the presence of a large introgression. Molecular markers identify the introgression as being from chromosome 6E. They also show the loss of chromosome 7E from BC_4-181B, reducing the number of whole *Th. elongatum* chromosomes present from three in BC_3-392A to two.

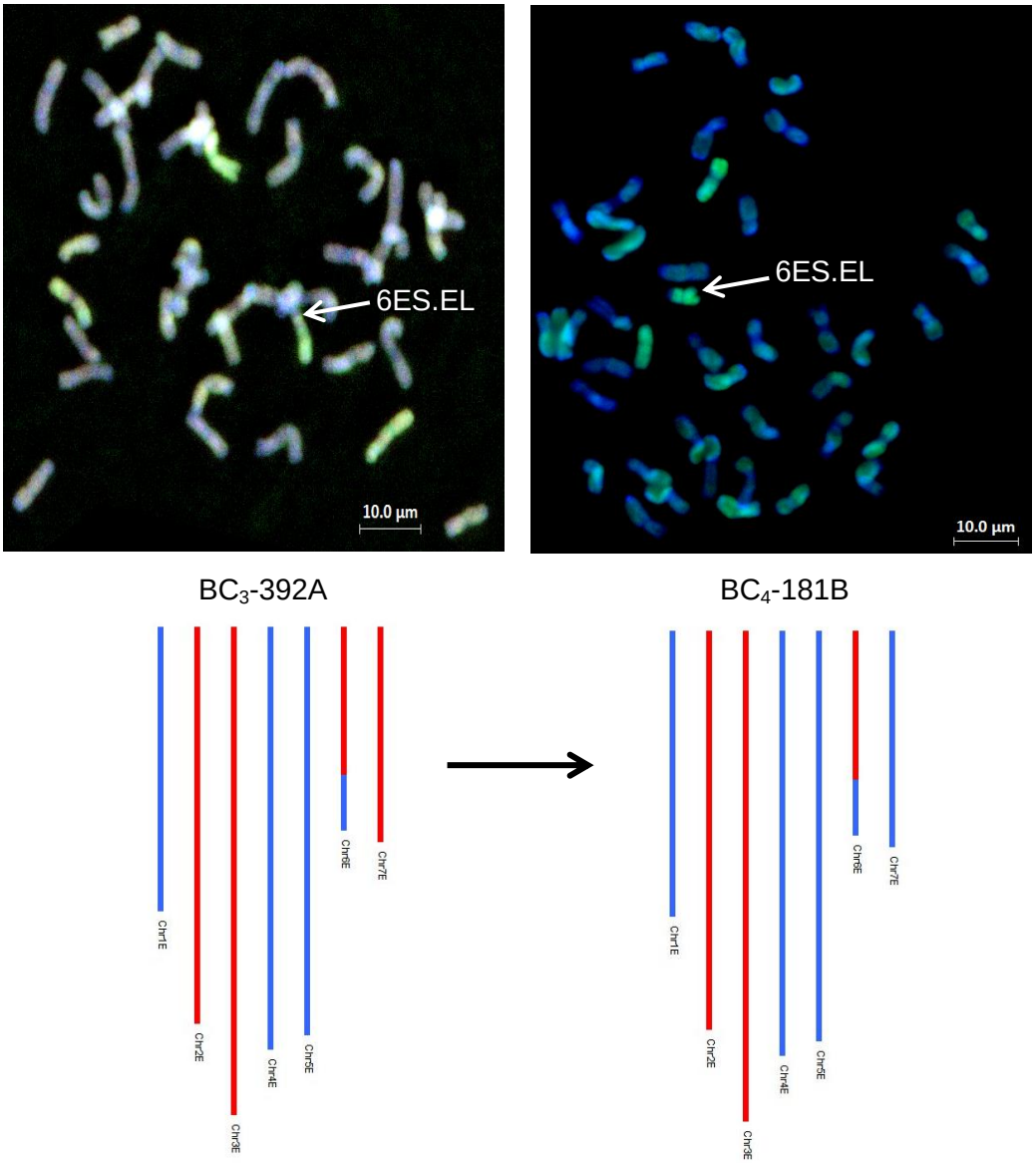


Figure 6.8: GISH and genotype results for BC_3-392A and BC_4-181B. GISH images show a large introgression in both lines. The molecular markers identify the introgression as 6ES.EL. The number of whole chromosomes from *Th. elongatum* 401007 reduced from three in BC_3-392A to two in BC_4-181B.

The BLAST results of the Affymetrix SNP markers showed that 89.4%, 92.4% and 94.5% of the markers had a significant BLAST hit on the A, B and D genomes of wheat respectively. From the BLAST hits, 25.9%, 41.1% and 39.9% of the markers had an overall top hit on the A, B and D genomes of wheat respectively (Appendix 9.6). Some markers showed the same score for the top hit for more than one genome. The D genome was used in this analysis as it had the highest significant BLAST hits (94.5%) and previous research has stated that the D genome of wheat and the E genome of *Thinopyrum* species was most similar (Liu, Li and Zhang, 2007). Comparative analysis using Circos v. 0.67 (Krzywinski *et al.*, 2009) of the loci on the genetic map of the E genome and their corresponding physical positions on the D genome of wheat (obtained through BLAST against the wheat reference sequence) showed that macro-synteny between the genomes is maintained (

Figure 6.9). The E and D genomes also maintain collinearity, except for in chromosome 7 where the E genome is inverted in comparison to the D genome. The Circos plot also clearly shows that *Th. elongatum* 401007 does not contain the 4/5 translocation seen in wheat. One loci from chromosome 4E maps to group 2 in wheat and a separate loci from chromosome 6E maps to group 5 in wheat, but there are no large translocations present.

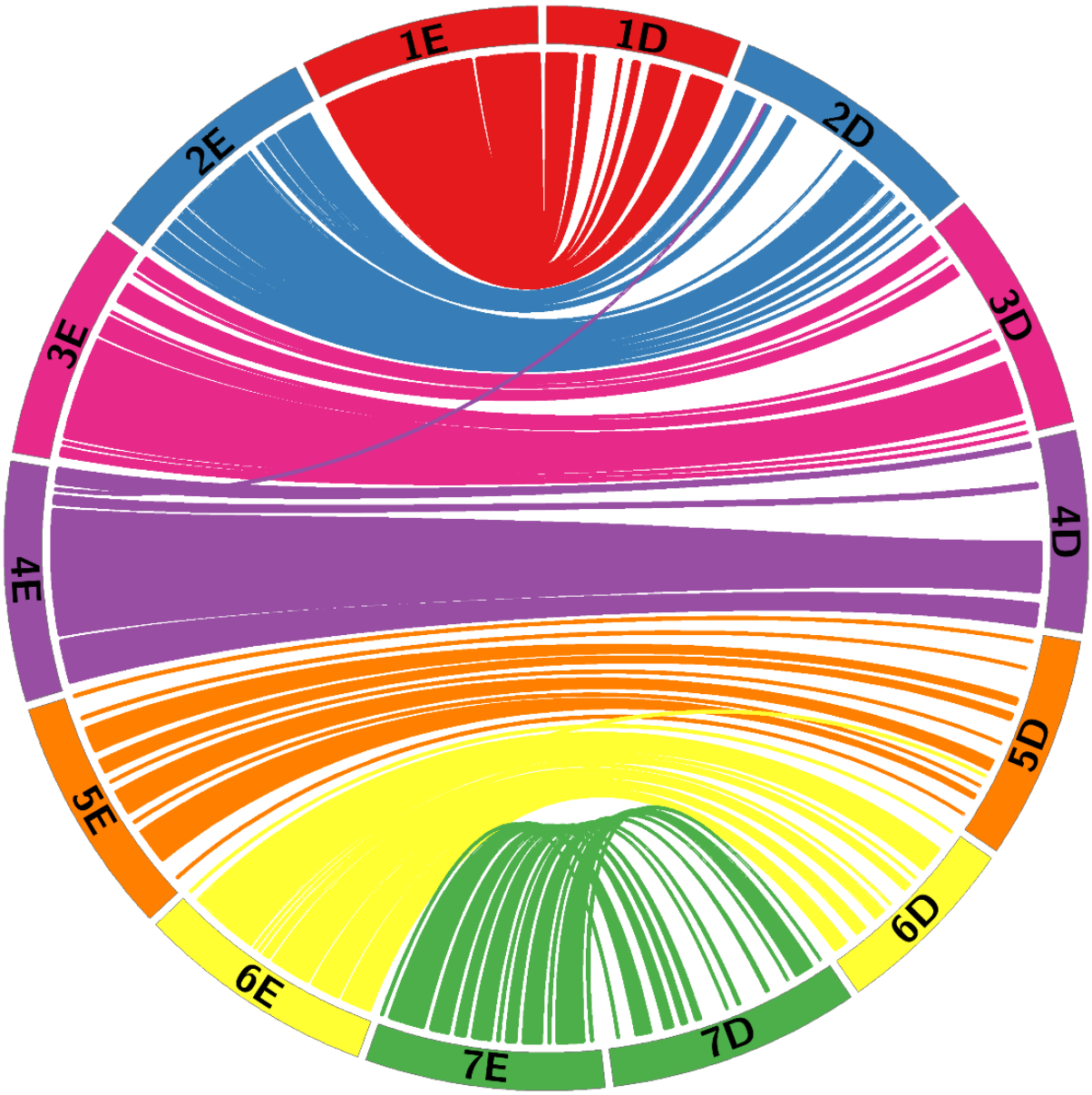


Figure 6.9: The synteny and collinearity between the E genome of *Th. elongatum* 401007 and the D genome of wheat; visualised by Circos v. 0.67 (Krzywinski *et al.*, 2009).

6.5. Materials and methods for KASP™ marker production

6.5.1. Primer design

To design the KASP™ markers, all Affymetrix marker sequences were put into a new BLAST search against the wheat IWGSC Survey sequence (IWGSC, 2014) This was for three purposes:

(i) to validate the presence of SNPs

(ii) to assign markers to specific chromosome positions via their IWGSC codes, and

(iii) to ensure markers covered a good spread of the genome.

The top scaffold position for each sequence (for those with a score above 90) was recorded and selected to progress. The genomic location, overlapping genes and the POPSEQ genetic bin (Chapman *et al.*, 2015) associated with each marker were also recorded when possible. Finally, the alignment of each marker was checked to ensure it was not between exons and was not at the end of a sequence. From the BLAST results, all those primers that had only one copy on any one sub-genome of wheat were selected for primer design. This enabled the assays to be genome-specific in wheat. From the validated sequences, primer design was carried out using Primer3 (web version 4.0.0, available at http://primer3.ut.ee/). The product size range was set to 500-550 base pairs in length. Designed primer sequences were sent to Eurofins UK for production.

6.5.2. Primer dilution

Primers received back from Eurofins UK were separated into forward and reverse primers. 500µl of de-ionised water was added to each primer to make a 100pmol/µl solution. For use in PCR, the forward and reverse primers were combined and diluted to 10pmol/µl. The 10x dilution for each primer stock was made as follows:

- 160µl of dH_20
- 20µl Forward primer
- 20µl Reverse primer

6.5.3. PCR of primers for SNP detection

Diluted primers (10x) were run in PCRs against four wheat cultivars (Chinese Spring, Paragon, Highbury and Pavon 76) and all of the ten wild relatives in the WRC back-crossing programme. The reagent mix was made as follows:

	X1 Sample (µl)	**X 96 samples (µl)**
dH_2O	8.25	792
dNTPs (2.5mM)	2.0	192
10 x PCR buffer	2.5	240
Taq Polymerase	0.25	24
10x Primer	2	192
DNA (50ng/µl)	10	960
Total volume	**25**	**2400**

25µl of the reaction mixture was pipetted into each of the 96 wells of the PCR plate using a multi-channel pipette. Completed plates run in a GeneAmp® PCR System 9700 (Applied Biosystems, California) with the following programme:

Step	**Temperature (ºC)**	**Time (minutes)**	
Denaturation	95.0	5.0	
	94.0	0.5	X10
Annealing	65.0*	0.5	
Elongation	72.0	1.0	
	94.0	0.5	X28
	60.0	0.5	
	72.0	1.0	
Final Extension	72.0	5.0	
Hold	18.0		

*temperature decreases by 1ºC per cycle.

6.5.4. Visualisation and electrophoresis of PCR products

PCR products were run on agarose gels. A 1.6% agarose gel was made by adding 4g of agarose to 250ml of 1xTAE buffer in a conical flask. The mixture was heated to dissolve the agarose and left to cool. 10µl of Ethidium bromide was then added and mixed. The mixture was poured into the mould and left to set.

PCR amplification products had 3µl of 5x loading dye (Yorkshire biosci LTD cat#151101) added for visualisation. 25µl of each PCR product was loaded into individual wells in the set gel. The first well contained 5µl of a 1kb DNA ladder (Bioline hyper ladder 1, cat#Bio33026).

Loaded gels were placed into electrophoresis tanks and run at 180V for 45-60 minutes. Bands were visualised and photographed under UV light by a Gel Doc (Syngene, India). Identified bands were then excised and cleaned using NucleoSpin® Gel and PCR Clean up kits (Macherey-Nagel, Germany).

6.5.5. Sequencing

The purified PCR products for 437 wheat/*Th. elongatum* lines (see Appendix 9.4) were sent for Sanger sequencing at Source Bioscience (http://www,sourcebioscience.com/) along with 5µl of the forwards and reverse primer. Some sequences were treated with Source Bioscience Secondary structure treatment solution (dGTP chemistry) due to the high G-C content of the samples sent.

6.5.6. Sequencing analysis

Sequences obtained from Source Bioscience were quality checked using Chromas lite (v2.6.2 – available at https://technelysium.com.au/wp/chromas/). Any sequences that did not give clear, large peaks at the SNP region were discarded. The remaining sequences for a single primer were aligned using GeneDoc (version 2.7 – available at https://genedoc.software.informer.com/2.7/) in order to look for SNPs between wheat and the ten wild relatives, including *Th. elongatum* 401007. The alignment of the sequences was checked for any large insertions or deletions as a quality check and any gap columns in the sequence were removed.

Once aligned and quality checked, common SNPs between as many of the ten wild relatives and the two wheat cultivars (Paragon and Chinese Spring) were identified. These SNPs were preferable for design purposes as they could be functional for the molecular analysis of multiple wild relatives of wheat used within the WRC breeding programme. If common SNPs were unavailable but individual SNPs of high quality between single wild relative species and wheat were present these were also recorded. All SNPs of interest were replaced with their IUPAC code (IUPAC SNP nomenclature) enclosed within square brackets for the target SNP within the Chinese Spring sequence. 50 base pairs either side of the target SNP were also marked. If any additional SNPs were present in this sequence they were also replaced with their IUPAC code, but without the square bracket. Wherever possible, sequences were selected without additional SNPs present.

Another BLAST search of the selected sequences was performed against the wheat RefSeq1 assembly (IWGSC, 2018). At this point the actual physical positions of the markers were recorded. Other copies of the same sequence were looked up on the same or other chromosome arms and noted down. This enabled possible chromosome arm specific markers to be identified from the non-chromosome arm specific markers. Other information recorded included: the POPSEQ genetic position for the target SNP, the chromosome arm location (if available), the wild relative species allele, the wheat allele, the SNP IUPAC code, the SNP position in GeneDoc and the ~100bp sequence containing the target SNP. It must be noted that due to the sheer scale of this work, it was done in collaboration with other PhD researchers and Research Fellows in the WRC group. I contributed to the primer design and validation of markers in chromosome group 1 and 5 of wheat. Other chromosome groups were covered by other members of the WRC group.

Marker sequences were then sent to LGC for KASP™ Assay design. LGC were also sent 5,000 DNA samples from back-crossed lines, including the 437 from the *Th. elongatum* breeding programme (see Appendix 9.4) to run the KASP™ Assays on.

After initial validation, the KASP™ Assay sequences of working assays were obtained from LGC and used to order KASP™ primers from Sigma for in-house genotyping. The 437 *Th.* elongatum back-crossed lines genotypes were received back from LGC and required further analysis.

6.5.7. KASP™ marker validation via mc-GISH

To validate the KASP™ markers, metaphase slides for 11 lines were produced using the protocol outlined in section 5.3.2. The 11 lines were:

- BC_2F_2-483B
- BC_2F_2-488A
- BC_2F_2-497A
- BC_2F_2-498A
- BC_2F_2-532D
- BC_2F_2-533D
- BC_2F_2-536B
- BC_2F_2-539A
- BC_2F_2-542A
- BC_2F_2-545A
- BC_2F_2-556A

These lines were selected as they were the only 11 out of the 437 genotyped using the KASP™ markers that had root tips available.

Each slide was then probed with the following probe mixture:

- 1.2µl of A genomic probe (*T. urartu)*
- 4.0µl of B genome blocking DNA *(Ae. speltoides)*
- 0.8µl of D genomic probe (*Ae. tauschii)*
- 1.0µl of *Th. elongatum* 401007 genomic probe
- 3.0µl of 2xSSC in 1xTE

Slides were probed as outlined in section 5.3.6.

6.5.8. 74x KASP™ marker primer dilutions

For use in the in-house genotyping platform, the forward, reverse and common primers (Sigma) for each KASP™ marker assay were combined and diluted to a stock concentration of 100mM (74x dilution). To make 500µl of stock solution the following solutions were combined in a pre-labelled Eppendorf tube for each marker:

- 395µl dH_2O
- 15 µl Forwards primer (100µM)
- 15 µl Reverse primer (100µM)
- 75 µl Common primer (100µM)

6.5.9. KASP™ marker validation via PCR

DNA extractions were performed on 89 back-crossed lines, three samples of *Th. elongatum* 401007 and three samples of Chinese Spring. DNA extractions were performed as outlined in section 3.2. The lines selected were chosen due to their GISH results showing they have a small number of segments. Samples were then loaded into a 96 well DNA source plate in the

layout shown in Table 6.3. One well was loaded with de-ionised water as a blank control. A subset of 84 markers, 12 from each chromosome, were selected that spanned each chromosome of wheat to test against each sample. The list of markers selected and their corresponding wheat chromosome is shown in Table 6.2.

Table 6.2: The WRC KASP™ markers selected to detect segments in back-crossed lines of wheat/*Th.elongatum* 401007 separated into their corresponding wheat chromosome.

Chr 1	Chr 2	Chr 3	Chr 4	Chr 5	Chr 6	Chr 7
WRC0002	WRC0148	WRC0293	WRC0439	WRC0563	WRC0712	WRC0866
WRC0022	WRC0159	WRC0308	WRC0460	WRC0575	WRC0726	WRC0871
WRC0033	WRC0171	WRC0316	WRC0466	WRC0588	WRC0739	WRC0895
WRC0044	WRC0190	WRC0336	WRC0476	WRC0607	WRC0753	WRC0898
WRC0049	WRC0198	WRC0345	WRC0482	WRC0614	WRC0760	WRC0907
WRC0059	WRC0207	WRC0360	WRC0492	WRC0625	WRC0774	WRC0913
WRC0077	WRC0225	WRC0377	WRC0504	WRC0645	WRC0785	WRC0929
WRC0095	WRC0244	WRC0389	WRC0514	WRC0658	WRC0804	WRC0941
WRC0103	WRC0255	WRC0391	WRC0518	WRC0668	WRC0811	WRC0954
WRC0114	WRC0262	WRC0404	WRC0531	WRC0684	WRC0822	WRC0962
WRC0125	WRC0275	WRC0420	WRC0542	WRC0698	WRC0832	WRC0983
WRC0135	WRC0289	WRC0435	WRC0557	WRC0706	WRC0851	WRC0998

Table 6.3: 96 well plate DNA Source plate layout for the 89 back-crossed lines, three samples of paragon, three samples of *Th. elongatum* 401007 and a blank well for a control.

Paragon 1	Paragon 2	Paragon 3	*Th. elong* 401007 1	*Th. elong* 401007 2	*Th. elong* 401007 3	BC_3-383A	BC_3-388E	BC_3-390D	BC_3-664A	BC_3-667B	BC_3-667E
BC_3-668B	BC_3-669A	BC_3-669B	BC_3-677A	BC_3-677B	BC_3-677C	BC_3F_1-224C	BC_3F_1-224D	BC_3F_1-227A	BC_3F_1-227B	BC_3F_1-229B	BC_3F_1-230D
BC_3F_1-231B	BC_3F_1-233B	BC_3F_1-233C	BC_3F_1-234B	BC_3F_1-234C	BC_4-172A	BC_4-172B	BC_4-172C	BC_4-176B	BC_4-176C	BC_4-179A	BC_4-179C
BC_4-179D	BC_4-181B	BC_4-183A	BC_4-191A	BC_4-239D	BC_4-245A	BC_4-245B	BC_4-245C	BC_4-249A	BC_4-249B	BC_4-250B	BC_4-250C
BC_3F_1-228B	BC_4F_1-100A	BC_4F_1-101A	BC_4F_1-101B	BC_4F_1-101D	BC_4F_1-103B	BC_4F_1-107C	BC_4F_1-108B	BC_4F_1-108C	BC_4F_1-108D	BC_4F_1-110A	BC_4F_1-110C
BC_4F_1-112A	BC_4F_1-112B	BC_4F_1-90A	BC_4F_1-90B	BC_4F_1-90C	BC_4F_1-91B	BC_4F_1-92B	BC_4F_1-92C	BC_4F_1-92D	BC_4F_1-94D	BC_4F_1-95D	BC_4F_1-99B
BC_5-69B	BC_5-73C	BC_5-74C	BC_5-75A	BC_5-76A	BC_5-76B	BC_5-79B	BC_5-83A	BC_5-83B	BC_5-87A	BC_5-87B	BC_5-87C
BC_5-88B	BC_5-88C	BC_5-88D	BC_5-90C	BC_5-90D	BC_5-91D	BC_5-93A	BC_5-93C	BC_5-94A	BC_5F_1-3A	BC_5F_1-4A	blank

With the 384 plate layout available, it was possible to test four markers per plate, with 96 wells needed for each marker (see Appendix 9.5 for the layout of Plate 1 as an example). 5µl of each DNA sample was loaded into the correct well of the 384 PCR plate from the DNA Source plate shown in Table 6.3. The DNA was then dried at 80°C for 30 minutes. 5µl of the PCR reaction mix was then added to each well. Primers used in the PCR master mix were diluted as stated in section 6.5.8. The PCR master mix for KASP™ marker analysis is shown below:

Reagent	**1x**	**96x (+30% extra)**
dH_2O	2.43µl	303.3µl
Rox PACE mix (see Appendix 9.1)	2.50µl	312µl
74x Primers (see section 6.5.8)	0.068µl	8.48µl
Total volumes	5µl	624µl

The PCR plates were ran on the ProFlex PCR system (Applied Biosystems) with the following programme:

Step	**Temperature (ºC)**	**Time (minutes)**	**No. of cycles**
Denaturation	94.0	15.00	
	94.0	0.25	X10
Annealing	65.0	1.0	
Elongation	94.0	0.25	X40
	57.0	1.0	
Final Hold Point	4.0	-	

Results from these PCRs produced allelic discrimination plots and were then scored and analysed using QuantStudio™ 5 Real-Time PCR System and QuantStudio™ Design and Analysis Software v1.4.3 (Applied Biosystems, California).

6.6. Results and discussion for KASP™ markers

6.6.1. Primer design results

From the Affymetrix sequence BLAST search, a total of 2,422 primers were designed for all ten of the wild relatives in the Nottingham BBSRC programme. These primers spanned across the entire genome of wheat. Table 6.4 shows how many primers were designed for each chromosome of wheat.

Table 6.4: The number of primers designed from Affymetrix marker sequence for SNP detection prior to KASP™ marker design.

Chromosome	Number of primers designed
1	261
2	401
3	428
4	255
5	290
6	309
7	478
Total	**2,422**

6.6.2. KASP™ marker design results

A total of 1,000 KASP™ markers were selected from across the wheat genome (from the possible 2,422 primers available) and assays were produced by LGC. This subset of markers was selected based on their distribution across the chromosome arms for each wild relative of wheat, aiming for a minimum of 5 per arm for each species. Where possible, markers common to multiple wild relative species were used. The number of markers designed for each chromosome of wheat is shown in Table 6.5.

Table 6.5: The total number of WRC KASP™ markers designed between all ten of the wild relative species and wheat.

Chromosome Arm	Number of KASP markers designed
1A	45
1B	56
1D	46
2A	48
2B	54
2D	43
3A	45
3B	53
3D	45
4A	46
4B	35
4D	43
5A	49
5B	56
5D	47
6A	45
6B	53
6D	49
7A	44
7B	47
7D	51
Total	**1,000**

The spread of markers across the whole genome was good, with chromosome 4B having the least number of markers (35) and chromosomes 1B and 5B having the most (56). This coverage of the genome should enable more accurate detection of introgression events.

These 1,000 markers were run by LGC on sequences from 5,000 genotypes from back-crossed lines from all ten of the wild relatives of wheat, including 437 lines from *Th. elongatum* back-crosses. Results were analysed using

SNPviewer (LGC) in order to check how many markers worked specifically for *Th. elongatum* 401007.

Table 6.6 shows the subgroup of these 1,000 markers that were designed to be capable of detecting the presence of *Th. elongatum* 401007 chromatin and the number of markers which were shown to actually detect *Th. elongatum* 401007 DNA. In total, 874 markers were produced that were theoretically capable of detecting *Th. elongatum* 401007 DNA across all chromosomes of wheat. The range of markers for each chromosome varied from a minimum of 33 markers for chromosome 4B to a maximum of 48 markers for chromosomes 1B and 5B. From these 874, 346 were successful in detecting the presence of *Th. elongatum* 401007 DNA.

Figure 6.10 shows a diagrammatic representation of the KASP™ markers designed for *Th. elongatum* 401007 in linkage group 1. The figure shows that, based on their synteny to wheat markers, they cover a good proportion of the linkage group and should therefore enable detection of introgressions more accurately.

Table 6.6: The number of WRC KASP™ markers designed to detect *Th. elongatum* 401007 introgressions, separated into each wheat chromosome arm.

Chromosome Arm	Number of KASP™ markers designed to detect *Th. elongatum* 401007	Number of KASP™ markers shown to detect *Th. elongatum* 401007
1A	38	11
1B	48	17
1D	43	20
2A	42	16
2B	47	25
2D	40	20
3A	41	8
3B	46	22
3D	40	16
4A	40	21
4B	33	9
4D	39	20
5A	44	16
5B	48	18
5D	39	18
6A	37	16
6B	47	18
6D	43	15
7A	36	13
7B	41	15
7D	42	12
Total	**874**	**346**

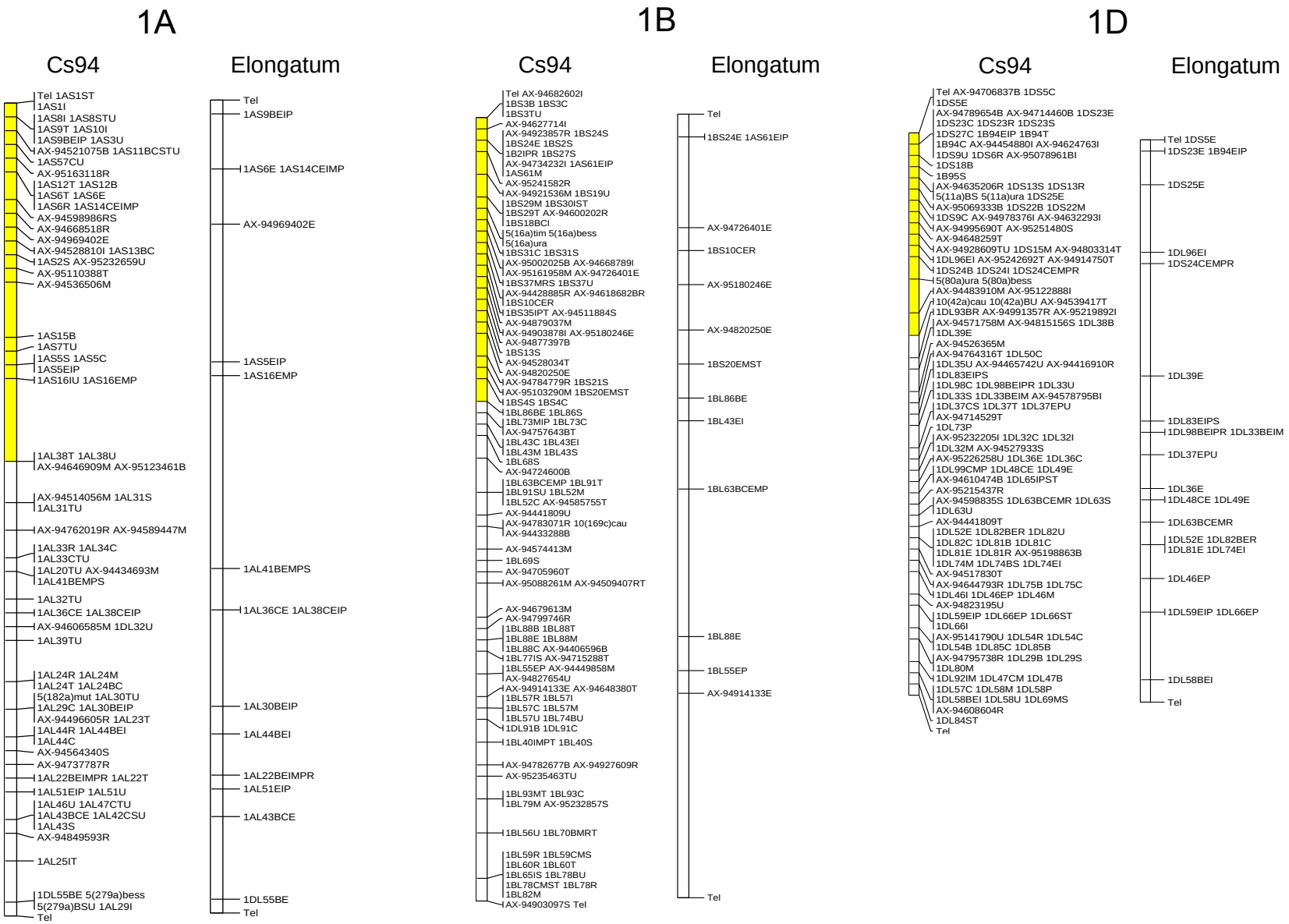


Figure 6.10: A rough map view of the KASP™ markers designed for Linkage Group 1 of *Th. elongatum* 401007 in comparison to Chinese Spring. The yellow segments represent the short arm of the chromosomes.

6.6.3. KASP™ marker genotyping results

From the 437 lines genotyped by LGC, 266 lines appeared to have homozygous *Th. elongatum* segments of varying sizes across all 7 wheat chromosomes. This work was the initial attempt at using the KASP™ markers to identify *Th. elongatum* segments in a wheat background, and as such validation of the markers is required before this number can be confirmed. Due to the quality of data observed, it was not possible to state the number of introgressions detected by the KASP™ markers.

6.6.4. KASP™ marker validation via Mc-GISH results

Mc-GISH experiments were carried out on 11 lines that had KASP™ marker genotypes available. Out of the 11 lines that underwent mc-GISH, 7 appeared to loosely match their KASP™ marker results (see Table 6.7).

Table 6.7: A table comparing the results from the KASP™ marker analysis with mc-GISH analysis

Line	Mc-GISH results	KASP™ marker results
BC_2F_2-483B	6 whole chromosomes	6 whole chromosomes
BC_2F_2-488A	4 whole chromosomes	4 whole chromosomes
BC_2F_2-497A	1 homozygous E to D introgression, a second introgression and 5 whole chromosomes	One introgressions and 5 whole chromosomes
BC_2F_2-498A	9 whole chromosomes	7 whole chromosomes
BC_2F_2-532D	8 whole chromosomes and a homozygous introgression	6 whole chromosomes and an introgression
BC_2F_2-533D	6 whole chromosomes	6 whole chromosomes
BC_2F_2-536B	Not clear (at least 4 whole chromosomes)	4 whole chromosomes
BC_2F_2-539A	Not clear (at least five whole chromosomes)	Five whole chromosomes
BC_2F_2-542A	4 whole chromosomes and an A to E homozygous introgression	4 whole chromosomes and an introgression
BC_2F_2-545A	7 whole chromosomes	7 whole chromosomes
BC_2F_2-556A	6 whole chromosomes and a homozygous B to E introgression	7 whole chromosomes

Figure 6.11 shows the KASP™ marker results and corresponding mc-GISH results for one of these 11 lines, BC_2F_2-488A. Whilst the two results do

loosely support each other, there are a number of KASP™ markers that need further investigation. The data used to produce these initial KASP™ marker results is from the first attempt and will undergo further validation to improve accuracy. As you can see the data shown in Figure 6.11 from the KASP™ markers is not as accurate or easy to interpret as the Affymetrix SNP markers, and therefore the results cannot be relied upon accurately.

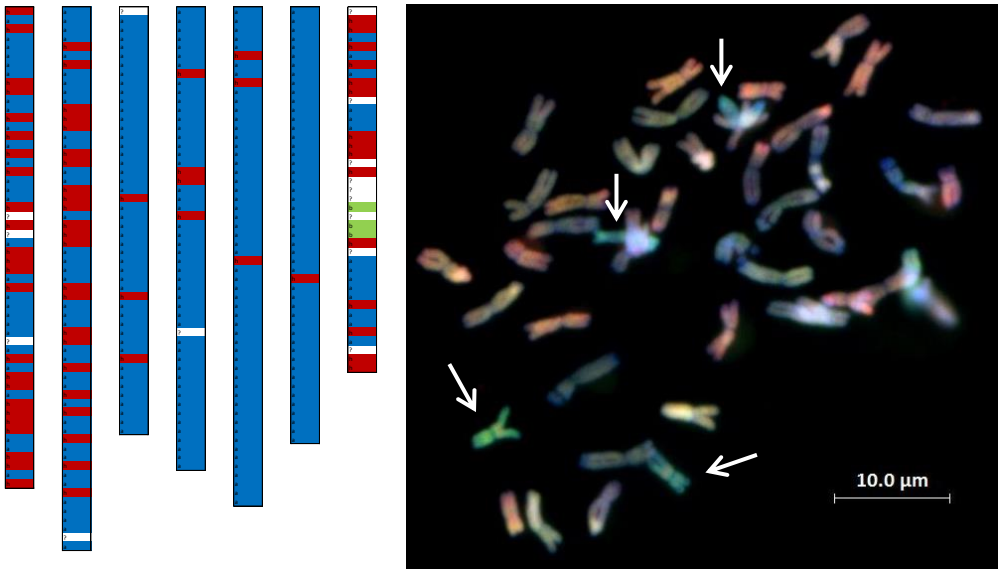


Figure 6.11: The KASP™ marker results and corresponding mc-GISH results for BC_2F_2-488A. The KASP™ marker results show a possible whole chromosome in Group 1, 2 and a possible homozygous whole in group 7. Red sections represent *Th. elongatum* 401007 and blue markers represent wheat. The green markers represent homozygous markers. Red markers on Groups 3, 4, 5 and 6 are possible faulty markers. White markers represent null calls. White arrows on the mc-GISH image indicate the four whole chromosomes that should be present in the KASP™ genotype.

6.6.5. KASP™ marker validation via PCR results

From the 84 markers selected for in-house genotyping, 67.9% (57) were shown to be capable of detecting heterozygous and homozygous segments from *Th. elongatum* 401007 in a wheat background. A total of 286 segments were detected by these markers from 89 lines. Results are shown in Table 6.8. It is important to note that out of the 89 lines chosen for analysis with this subset of KASP™ markers; only 11 of the results matched their corresponding GISH results. Further work will be required to validate these

results and resolve the discrepancies between cytogenetic and molecular analysis of lines.

Table 6.8: The number of segments detected by the subset of 84 KASP™ markers selected, divided into the chromosome arm each marker was designed for.

Chromosome Arm	Number of markers tested	Number of markers working	Number of lines with segments detected
1A	4	4	17
1B	4	3	11
1D	4	2	13
2A	4	4	35
2B	4	2	12
2D	4	3	18
3A	4	3	15
3B	4	2	9
3D	4	2	13
4A	4	4	24
4B	4	0	0
4D	4	2	12
5A	4	4	16
5B	4	3	4
5D	4	4	14
6A	4	3	7
6B	4	3	16
6D	4	3	23
7A	4	2	0
7B	4	2	2
7D	4	2	25
Totals	84	57	286

The discrepancies between the GISH and KASP™ results could be due to a number of factors. Firstly, the subset of markers selected may not have covered the area of the chromosome where *Th. elongatum* 401007 chromatin was present and therefore some introgressions may not have been detected. Secondly, the decaploid nature of *Th. elongatum* 401007 implies a high proportion of repetitive DNA sequences, potentially resulting in primers

annealing to multiple sequences. This could explain why some lines that were shown with GISH to have single segments hybridised with primers from multiple chromosomes. This anomaly could also be explained by unknown translocations in the *Th. elongatum* 401007 genome. A final factor affecting the quality of these results could be the quality of the DNA used in the process. Repetition of these experiments would have been exceptionally useful for validation, however was not possible due to time constraints. Whilst not matching the GISH results these preliminary KASP™ results offer some interesting insights, if observed from a qualitative stance.

Three sibling lines, BC_4F_1-101A, BC_4F_1-101B and BC_4F_1-101D, showed a single, large introgression when investigated using GISH. For this family, the KASP™ markers suggest that this introgression is from linkage group 2. Further testing with additional KASP™ markers would help validate this finding. Results obtained so far for these 'sibling' lines are shown below (Table 6.9).

Table 6.9: KASP™ marker results for 'sibling' lines BC_4F_1-101A, BC_4F_1-101B and BC_4F_1-101D

Line	KASP™ Marker ID	Marker Chromosome Arm
BC_4F_1-101A	WRC0159	2A
	WRC0275	2D
BC_4F_1-101B	WRC0159	2A
	WRC0275	2D
BC_4F_1-101D	WRC0159	2A
	WRC0275	2D

However, for another sibling line, the KASP™ markers do not correspond to their GISH or the Affymetrix SNP marker results. BC_4-172A, BC_4-172B and BC_4-172C were all shown to have a single introgression, approximately half the length of the chromosome. The KASP™ marker results for these three lines are shown below (Table 6.10).

Table 6.10: The KASP™ marker results for the sibling lines BC_4-172A, BC_4-172B and BC_4-172C

Lines	KASP™ Marker ID	Marker Chromosome Arm
BC_4-172A	WRC0044	1A
	WRC0404	3D
	WRC0832	6D
	WRC0998	7D
BC_4-172B	WRC0044	1A
	WRC0404	3D
	WRC0832	6D
BC_4-172C	WRC0044	1A
	WRC0404	3D
	WRC0832	6D
	WRC0998	7D

BC_4-172A and BC_4-172C produced identical results and bound to KASP™ markers from chromosomes 1, 3, 6 and 7. BC_4-172B bound with three identical markers for chromosomes 1, 3 and 6, but didn't bind to WRC0998 (chromosome 7). These results show that the KASP™ marker work still needs improving, as the Affymetrix SNP markers clearly showed the introgressions came from chromosome 7. The fact that sibling lines are binding to identical markers indicates that the DNA is working correctly. This result could possibly be evidence to support the presence of unknown translocations or highly repetitive sequences in the genome of *Th. elongatum* 401007. However, this seems unlikely from the comparative analysis of the Affymetrix SNP markers showing no large translocations (

Figure 6.9).

The KASP™ markers are working within the in-house genotyping system. Figure 6.12 and Figure 6.13 show the allelic discrimination plots for the two markers, WRC0476 (linkage group 4) and WRC0962 (linkage group 7). Both plots show clear distinct groupings allowing heterozygous lines to be identified. BC_3F_1-234C is indicated using a black arrow on both figures. The GISH results for this line show a clear, single introgression, however the two markers are from different linkage groups. This could suggest the presence of

a possible translocation in *Th. elongatum* 401007. Further work with more of the markers will be required to investigate this.

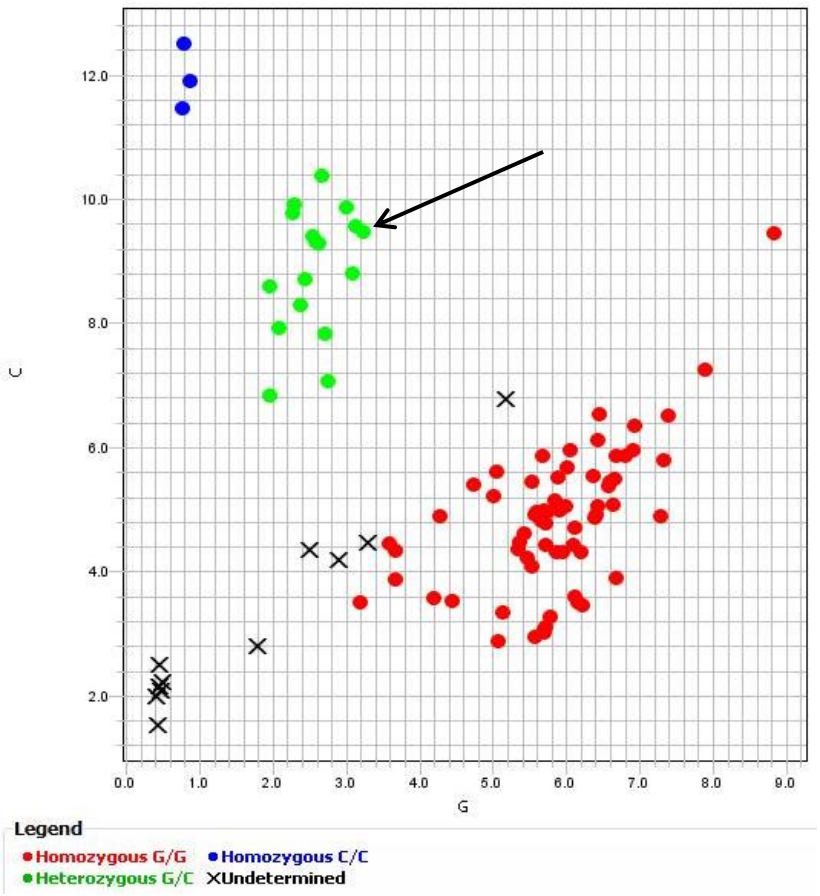


Figure 6.12: The Allelic Discrimination Plot for marker WRC0476 (chromosome 4A) after 70x PCR cycles. The three blue lines represent the three *Th. elongatum* 401007 controls, the three wheat controls are within the red cluster and green dots represent heterozygous lines. Black crosses represent lines which cannot be determined. Line BC_3F_1-234C is indicated with a black arrow.

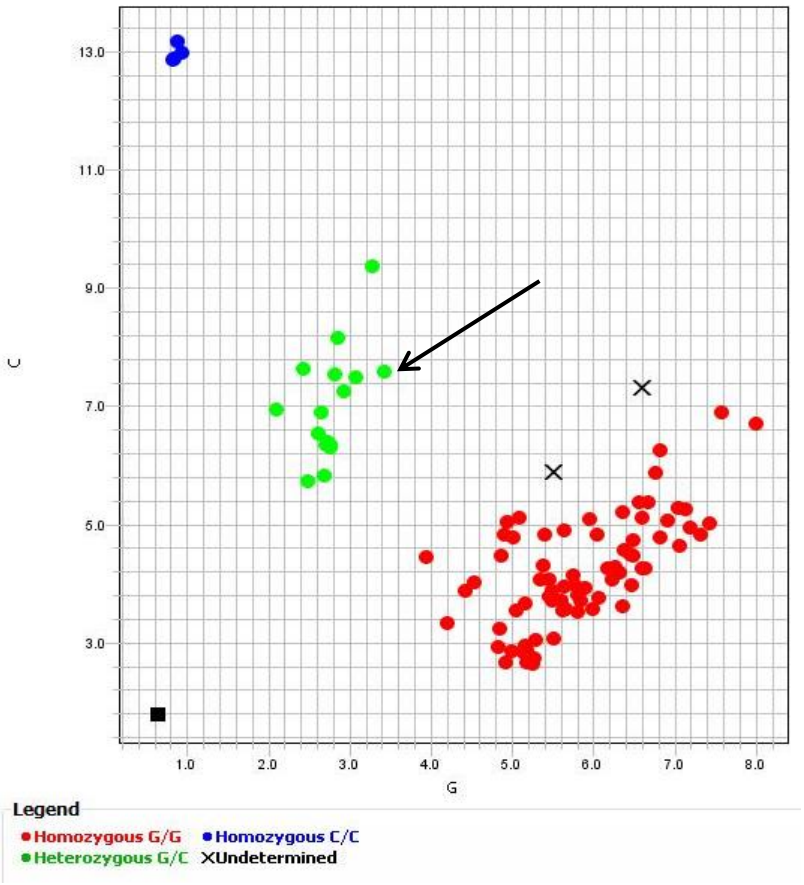


Figure 6.13: The Allelic Discrimination Plot for marker WRC0962 (chromosome7D) after x70 PCR cycles. The three blue lines represent the three *Th. elongatum* 401007 controls, the three wheat controls are within the red cluster and green dots represent heterozygous lines. Black crosses represent lines which cannot be determined. The black square indicates the blank control. Line BC_3F_1-234C is indicated with a black arrow.

To summarise the molecular marker work, the KASP™ markers show potential to identify the presence of *Th. elongatum* 401007 segments that are heterozygous and homozygous in a wheat background. However, they currently are not matching the pre-existing cytogenetic results or the validated Affymetrix SNP markers done on the same lines. This does not mean the KASP™ markers are not usable, however it does mean they require significant refinement and validation. Future work should include using a larger subset of KASP™ markers on single segment lines to investigate the presence of translocations or repetitive sequences. Since the completion of the work within this thesis, the KASP™ marker protocol has already been revised and results have shown improvement. The quality of DNA used in the PCRs should also be investigated to rule it out as a variable within the results. The use of iso-propanol to improve the DNA extraction of samples was being investigated as a way to improve the DNA quality used in the PCRs, however could not be used for this work due to time constraints. The segments identified by KASP™ markers should be tracked in future progeny with the same markers to investigate any reduction in size or loss of segment.

The Affymetrix SNP markers are a functioning, high-throughput method that can be utilised in future work involving *Th. elongatum* 401007. The Affymetrix SNP markers were supported and validated by GISH work completed on back-crossed lines produced in the wheat/*Th. elongatum* breeding programme. They enabled introgressions to be assigned to specific chromosomes and a putative genetic linkage map to be produced.

7. Final Discussion and Conclusions

The Generation of Germplasm

Securing and improving upon all crop yields for future generations will require the generation of cultivars that can match or exceed demand whilst overcoming the increasing challenges posed by climate change and the increasing human population (Gustafson, Borlaug and Raven, 2010). The introduction of genetic variation from crop wild relatives which are known to contain many biotic and abiotic traits of interest is one pathway to achieve such yield gains. Crop species that already contain genetic material from wild relatives include wheat, tomatoes, lettuce, sunflower, rice, maize, barley, sorghum, potato, lentil, banana and groundnut (Hajjar and Hodgkin, 2007). However, most of the traits transferred have focussed on pest or disease resistance and have not contributed to any increase in yield or an improved tolerance to climate change needed to meet future food demand (Hajjar and Hodgkin, 2007).

With wheat being an essential crop for global food security, the utilisation of crop wild relative variation such as that found in *Th. elongatum* is becoming increasing important to avoid future famine events. *Th. elongatum* is well established as a species with many disease and pest resistant genes, in addition to salinity and drought tolerance, a perennial growth habit and an increased photosynthetic efficiency (Fu *et al*. 2012; Fedak & Han 2005; Lammer *et al*. 2004; Reynolds *et al*. 2001; see section 4.1.1). The success of the breeding programme to introduce *Th. elongatum* traits into a wheat background remains to be investigated; however, the breeding programme has been overwhelmingly successful in producing back-crossed lines between the two species with genomic material from both species present.

The work outlined within this thesis is only the first step, yet it has successfully created over 8,400 back-crossed seed and over 55,600 self-fertilised seed from a minimum of 1,332 individual plants. The plants produced in the early back-cross stages (F_1 and BC_1) presented with an excellent fertility rate, far higher than originally hypothesised, which enabled rapid production of a large number of BC_2 seed. The seeds produced also had an excellent germination rate, again far higher than hypothesised, resulting in minimal loss of variation produced in the initial F_1 gametes. These increased fertility and germination rates could suggest a close synteny

between the genomes of wheat and *Th. elongatum* as such that when the initial F_1 gametes are formed, they are viable. Another possibility is that the high ploidy of the particular *Th. elongatum* accession used (401007) resulted in homoeologues of functional genes being present in any gametes formed. Any developmental genes lost from wheat chromosomes which were replaced by *Th. elongatum* chromosomes would be masked by the action of their homoeologues and therefore overall development would be unhindered. The fertility rates at such early stages of the breeding programme was unique to lines produced from *Th. elongatum* 401007, suggesting this particular wild relative of wheat is an excellent candidate for supplementing the genetic variation within the wheat genome (King *et al.*, 2017; Grewal *et al.*, 2018; Heath, 2018).

The wide range of phenotypes produced throughout this breeding programme was not possible to present quantitatively due to the lack of replicates and the sheer range in phenotypic traits that were presented. However, Figure 7.1 gives an example of the chlorophyll content variation in a very small subset of the population grown in August 2016 and Figure 7.2 shows the range in plant height that was observed in the population grown in January 2017. Due to the lack of replicates, it is not possible to correlate any physiological traits to any introgressed segments detected in these lines. It was unequivocally clear that phenotypic traits from *Th. elongatum* 401007 were been transferred to the hybrid lines. The lines presented with a large range in height, with the smallest recorded being just 35cm in height and the largest being 135cm tall (Figure 7.2). The shape and structure of the ears produced ranged from the long, thin, alternating spikelets of *Th. elongatum* to the shorter, stockier higher-yielding ears of wheat. The leaves varied in their width, colouring and thickness, with those being more *Th. elongatum* in their nature presenting as thinner, thicker, grooved and a darker green in colouring. The entire plant architecture could vary between the bushier *Th. elongatum* shape, or the more spaced out canopy of wheat. Whilst these traits may not be advantageous for breeders or future agronomic practice, it did prove beyond doubt that the hybrids being produced contained a mixture of genomic material from wheat and *Th. elongatum* 401007.

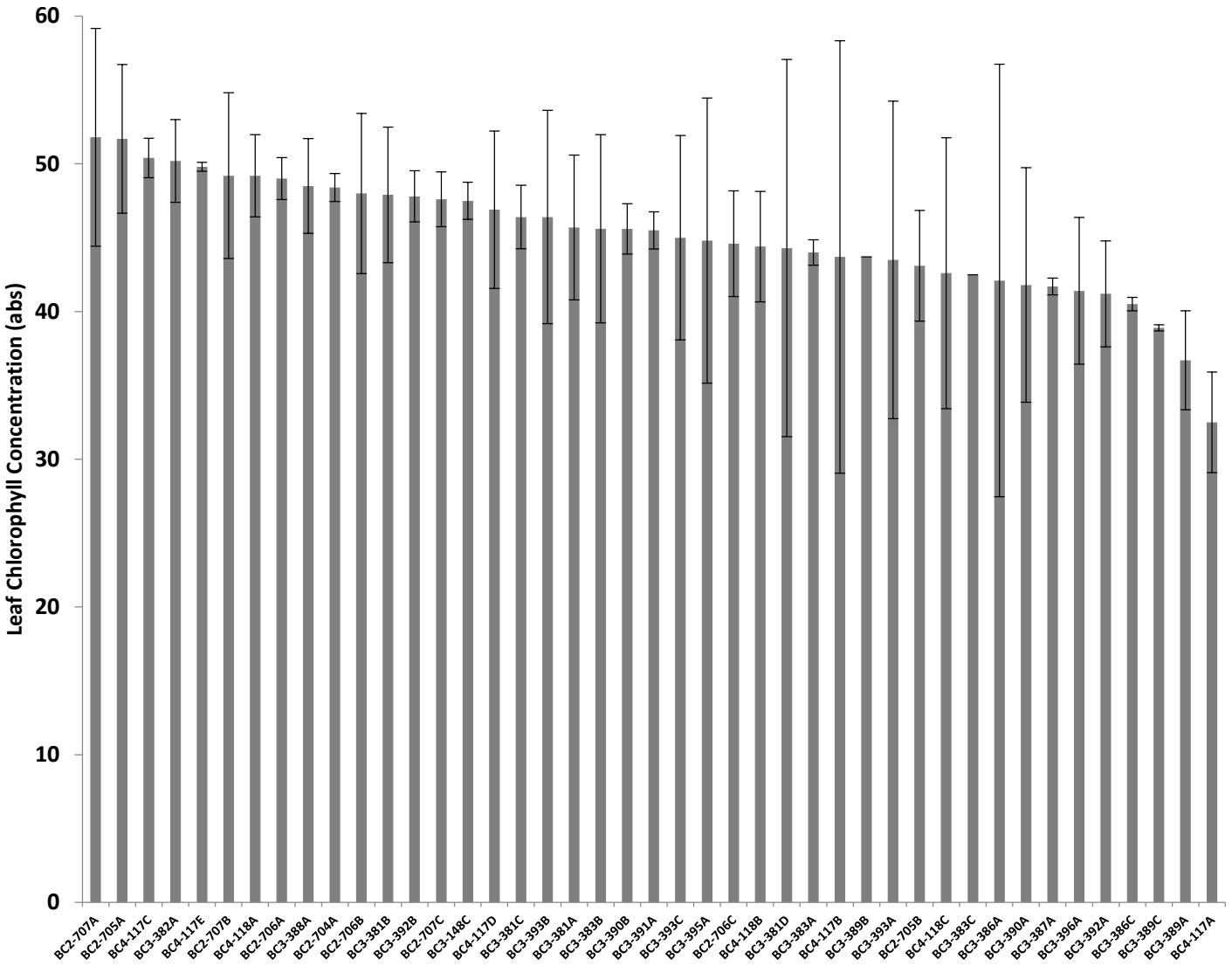


Figure 7.1: A brief insight into the average leaf chlorophyll concentration (measured by SPAD-502 (Minolta)) of a subset of the population grown in August 2016. Error bars display standard error of deviation (N=3).

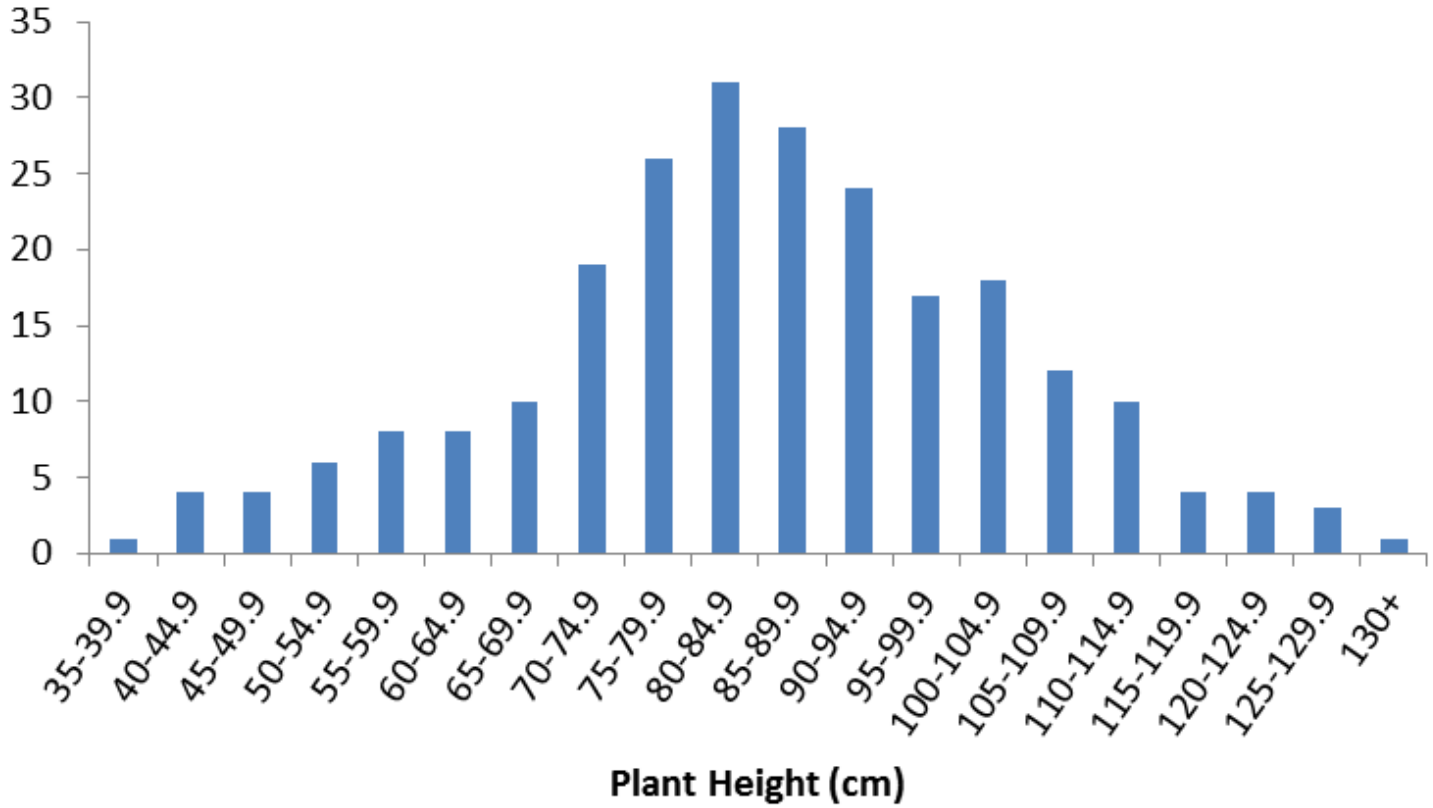


Figure 7.2: The range in plant height (cm) of the population grown in January 2017.

Phenotypic studies were not undertaken in this body of work due to time constraints and the lack of replicates. Future work done by breeders should include increasing the seed numbers of lines for replicates and subsequent screening for traits of agronomic interest including the aforementioned biotic and abiotic factors known to be present in *Th. elongatum*. Further breeding will also be required to reduce the number of segments present in each line in order to link any traits of interest to a particular segment from *Th. elongatum*. It was hypothesised for all ten of the wild relatives within the breeding programme that between two and four rounds of back-crossing would enable sufficient segregation of segments to produce a large number of plants with individual segments from the wild relative in a wheat background which spanned the entire genome of wheat. However, in practice, the lines produced in the breeding programme with *Th. elongatum* were shown via GISH to have a much higher number of segments than previously hypothesised. This is in spite of the back-crossed lines in the *Th. elongatum* breeding programme being taken through to the BC_5 generation. The number of segments was also much slower to decrease than expected. This finding could be due to the sheer number of whole chromosomes transferred from the decaploid *Th. elongatum* into the F_1 and subsequent BC_1 populations. The maintained number of whole chromosomes from *Th. elongatum* in a wheat background could indicate an increased synteny between the genomes of *Th. elongatum* and wheat resulting in the wild relative chromosomes facing a lower than expected selection pressure during meiosis.

This resulted in just 19 plants produced with single segments. The low number of plants with single segments made validating the subsequent genotyping challenging. Ideally, plants with single segments would have identified markers that corresponded to said segment and helped to eliminate any markers that were invalid for all other subsequent lines. Whilst the GISH images obtained for lines gave clear clarification on the number of segments present, they could not show which chromosomes the segments were in, and therefore if more than one segment existed in a line it became difficult to clarify which markers were correct and which were faulty.

Regardless of the difficulty in characterisation via molecular markers, the production of these 19 plants with single segments represents 19 opportunities for the improvement of current elite wheat cultivars. The wheat/ rye 1BL.1RS translocation has improved disease and drought tolerance in

over 200 commercial wheat cultivars for over the last 30 years (Schlegel and Korzun, 1997). With appropriate phenotypic testing and further breeding to reduce segment size where necessary, it is entirely possible that these 19 lines could contain an agronomic trait as beneficial to future wheat yields as the 1BL.1RS was to improving the yields in the past. Time and cost are two obstacles that would need to overcome for this to be achieved, as realistically, another 4-5 years further breeding would be needed to reduce linkage drag and confirm the stability of the segments in the population. Such an investment is usually too much for most private sector based plant breeding programmes (McCouch, 2013). However, recent initiatives such as Diversity Seek (Div-Seek) and the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) have been launched for improving the gene pools of crop species using their wild relatives and have increased the funding for future work sizeably (McCouch, 2013; Dempewolf *et al.*, 2014).

Cytogenetic Discussion

The work done using single-colour GISH within this thesis to identify *Th. elongatum* segments in a wheat background can be considered an overwhelming success. From 372 lines available from one crossing season, 327 successfully underwent the sc-GISH protocol. It was initially hypothesised that the number of introgressions would be fairly moderate, due to the reported homology between the E genome of *Th. elongatum* and the D genome of wheat (Liu, Li and Zhang, 2007). However, the final results showed a higher number of introgressions produced than expected, with a total of 143 introgressions detected, alongside an additional 660 whole chromosomes transferred from *Th. elongatum*. This number of introgressions detected exceeded expectations and suggests that the genomes of wheat and *Th. elongatum* 401007 may be more closely related than previously known. This also supports the hypothesis that *Th. elongatum* 401007 is an excellent candidate for improving the genetic variation in the wheat gene pool and will readily transfer its traits into a wheat background. The risk of linkage drag from *Th. elongatum* is an obvious potential limitation; however, phenotypic assessment via breeders will be required once genetically stable, single segment lines are produced. The GISH methodology, whilst time-consuming and laborious, was a consistently high-quality way to show the presence of *Th. elongatum* 401007 genetic material in the back-crossed lines genomes. The process was sped up midway through the work thanks to the

introduction of the fully automated Zeiss Axio Imager Z2 scanning microscope system automating the locating and photographing of metaphase spreads, however it can still be considered a low throughput way of detecting introgressions in comparison to molecular markers.

From reading previous literature on the *Thinopyrum* species, it became apparent that the nomenclature was an issue. The genomic constitution of polyploid accessions was also not clarified, partly due to the high homology between the E and J genomes dividing researchers as whether to classify them as the same or different genomes (Wang and Lu, 2014; Guo *et al.*, 2016). Another issue was that most previous research also focussed on the diploid species within the family, *Th. elongatum* and *Th. bessarabicum*, with very little phylogenetic or genomic work done on species or accessions with higher ploidy numbers. With this in mind, an investigation into the genomic constitution of the decaploid *Th. elongatum* 401007 was undertaken. Initially, sc-GISH images were completed on a diploid accession of *Th. elongatum* (acc. 531718). During these experiments, total genomic DNA probes from both the J and E genomes were shown to hybridise with the chromosomes from the diploid. Subsequent use of the probes on metaphase spreads of *Th. elongatum* 401007 showed the same result. Therefore, this body of work would support the hypothesis that the J and E genomes could be classified as modified versions of each other.

The V genome from *D. villosum* was also investigated due to its presence within the genome of *Th. intermedium* (Mahelka, Kopecký and Paštová, 2011; Mahelka, Kopecký and Baum, 2013); however, the GISH results from both the diploid and decaploid accessions of *Th. elongatum* showed that the V genome is not present. Therefore, this work can be used as evidence to rule out *D. villosum* as a progenitor of the decaploid accessions of *Th. elongatum*. Total genomic DNA probes were also made from *Ps. stipofolia* (St genome) and *Ae. speltoides* (B genome) and both were tested individually on the diploid accession of *Th. elongatum*. Both tests revealed that neither the B nor the St genome is present in the diploid accession of *Th. elongatum* as hypothesised. These steps were completed as a quality control and to investigate the homology of the genomes within the diploid accessions of *Th. elongatum*. Once concluded with the diploid, the same probes were used to investigate the genomic constitution of the decaploid accession *Th. elongatum* 401007. It was originally hypothesised that this particular

accession would contain two or at most three genomes (if the E and J genomes were counted separately). Previous literature suggested that these genomes would be the E, J and St genomes. Unexpectedly, the results from this work in fact showed the potential presence of sequences from 6 different genomes within the genome of *Th. elongatum*. As expected, the E, J and St genomes all bound to the chromosomes from *Th. elongatum* 401007. An interesting side note was that the St genome was only ever present as a centromeric region of an E (or J) chromosome – there were no whole St chromosomes present in the genome of *Th. elongatum* 401007 unlike *Th. intermedium* which has been shown to contain whole St chromosomes. The results also showed, for the first time reported as far as known, the presence of B genomic sequences in the genome of *Th. elongatum* 401007. This was unexpected, as it has previously been reported that the E genome from *Th. elongatum* and the D genome of wheat were highly homologous, yet the B genome has been reported to be the most distantly related from the genome of *Th. elongatum* (Liu, Li and Zhang, 2007). Rather than the B genome actually being a progenitor to the genome of *Th. elongatum,* it is far more likely that the B genome contains highly similar sequences to the J/E genome – as Figure 5.12 and Figure 5.13 show that it binds to the same segments of the chromosomes. This result suggests that the relationship between the J/E and the B genome of wheat is much closer than previously known.

Following this discovery, genomic probes were made from *T. urartu* (A genome) and *Ae. tauschii* (D genome). Both probes were found to bind to the chromosomes of *Th. elongatum*, although not as strongly as the B genomic probe. Once again, they bound to regions of the chromosomes that the J genome also hybridised with, suggesting that they may actually just be binding to conserved sequences between the two genomes. This result suggests that the B genome is most closely related to the J/E genome, followed by the A genome and D genome. Finally, a total genomic DNA probe was made from *S. cereale* (R genome) but was shown to not bind to *Th. elongatum* 401007 chromosomes and was therefore ruled out from its genomic constitution.

Therefore, as it currently stands, this research suggests that the following genomes may play a role in the genomic constitution of *Th. elongatum* 401007:

- E genome (*Th. elongatum* – diploid accession)

- J genome (*Th. bessarabicum*)
- St genome (*Ps. stipofolia*)

The following genomes were shown to have sequences present in the *Th. elongatum* 401007 genome but are not thought to be directly involved:

- B genome (*Ae. speltoides*)
- A genome (*T. urartu*)
- D genome (*Ae. tauschii*)

There is currently no clear answer from this work regarding the final constitution of the genome of *Th. elongatum* 401007 and further work would need to be done to resolve this matter. This work has shown that *Th. elongatum* 401007 is not an autopolyploid and does not contain any entire St chromosomes, and therefore, the most likely genomic constitution is either $JJJJJJJ^{St}J^{St}J^{St}J^{St}$ or $EEEEEEE^{St}E^{St}E^{St}E^{St}$. Future work could investigate the presence of previously untested genomes within the genome of *Th. elongatum* 401007 as in each mc-GISH experiment the probes left unbound sites along the chromosomes, suggesting the possible presence of a currently unidentified progenitor species.

The findings from the *Th. elongatum* 401007 genomic investigation raised questions regarding the cytogenetic and molecular marker work done on the introgression lines. Firstly, could any mc-GISH work done on back-crossed lines be missing segments due to potential missing genomes used to probe? Secondly, are the markers designed to detect *Th. elongatum* 401007 reliable based on the presence of common sequences between the E/J genome and the genomes which are also present in wheat? The sc-GISH results would not be affected by the genomic constitution of the parent, as the probe used to highlight introgressed segments was made from the total genomic DNA of *Th. elongatum* 401007 itself. Likewise the actual detection of the SNPs via exome capture would not be affected by these findings. The validation and reordering of markers into their subsequent linkage groups could be affected.

The mc-GISH research undertaken within this body of work utilised a ‘two-pronged’ approach. Initially, it was used to increase the amount of information obtained about recombinants. By using the J and St genomic probes it became possible not only to identify recombinants but also to classify which genomes were being transferred into the wheat background. Despite the parental GISH investigation showing the potential presence of sequences

from 6 different genomes within *Th. elongatum* 401007, this method seemed to reproduce similar results to sc-GISH using just the J and St genomic probes. This supports the hypothesis that there are actually only two genomes present in *Th. elongatum* 401007 (E/J and St) and the other genomic probes which bound (A, B and D) are only binding to conserved sequences at regions along the length of the *Th. elongatum* chromosomes. 32 lines were probed with the J and St genomes, of which 28 were successful and all 28 of the mc-GISH images matched their corresponding sc-GISH image in terms of number of recombinants and whole chromosomes from *Th. elongatum* 401007. Therefore, the use of total genomic probes from *Th. bessarabicum* and *Ps. stipofolia* to identify and characterise introgressions from *Th. elongatum* 401007 in a wheat background can be considered a successful approach to use in future studies.

The second mc-GISH approach included within this research utilised the A, B and D genomic probes to investigate with which genome of wheat recombinants were most likely to form. Previous literature research reported a high synteny between the E genome of *Th. elongatum* and the D genome of wheat (Liu, Li and Zhang, 2007). Therefore, it was hypothesised that the majority of recombinants would be within the D genome. However, the results showed the complete opposite. Out of 32 introgressions, just 4 were with the D genome, 11 with the B genome and 13 with the A genome. The remaining 4 introgressions were a range of double recombinants involving the A, D and E genomes. Considering the GISH results from the genome investigation of *Th. elongatum* 401007 this is not so surprising. The B genome was shown to clearly hybridise to the *Th. elongatum* 401007 genome and therefore could enable more frequent recombination events. This body of work suggests that for the decaploid *Thinopyrum* species, there is an increased synteny than previously recorded to the A and B genomes of wheat, and a reduced synteny to the D genome of wheat. Supporting results have been recently published in research on wheat/*Th. bessarabicum* recombinants (Grewal *et al.*, 2018). From 13 recombination events detected, 7 were with the B genome, 4 with the A and just 2 with the D genome of wheat.

This increased synteny of the E/J genome with the three genomes of wheat could explain why the recombination events appeared to occur along the length of the chromosomes rather than being limited to the telomeres or causing centromeric breaks.

Molecular Marker Discussion

The molecular markers were shown to have mixed success within this body of work. The Affymetrix SNP markers were successfully validated and were highly effective at detecting the presence of *Th. elongatum* DNA in a wheat background. However, the KASP markers were shown to produce variable results that require future work to improve accuracy and validity. This work is already underway but could not be included in this thesis due to time constraints. The Affymetrix SNP markers detected 202 introgressions across all seven linkage groups from 352 lines using 263 markers. The markers were also used to produce the first known genetic map for a decaploid *Th. elongatum*. When compared with the cytogenetic GISH images, the Affymetrix markers matched with all lines checked so far. This enabled the identification of introgressions and whole chromosomes transferred from *Th. elongatum* into a wheat background (see Figure 6.6 and Figure 6.7). It also enabled the short arm of long arm of the chromosome transferred to be identified. Once verified, the markers could then be used to identify the presence of introgressed segments and whole chromosomes in lines which had no previous cytogenetic or molecular characterisation. The Affymetrix SNP markers were also used for comparative analysis between the E genome and D genome of wheat (Figure 6.9). This work showed the macro-synteny and collinearity are maintained across the two genomes, with the exception of chromosome 7E which is inverted in comparison to chromosome 7D. This work also showed that the E genome does not contain the 4/5 translocation seen in wheat. Whilst these markers currently appear to be accurate and matching the GISH results, further lines must be checked to confirm validity.

The KASP™ markers were not as successful as the Affymetrix SNP markers at reliably detecting introgressions from *Th. elongatum* 401007 in a wheat background. In total, 346 KASP™ markers, with a good spread across the genome, are available to detect the presence of *Th. elongatum* 401007 genomic material. These markers are also capable of differentiating between heterozygous segments and homozygous segments (Figure 6.13). From the 455 lines analysed, a total of 266 homozygous segments were detected across the 7 chromosomes of wheat, however the subsequent validation of the markers suggests that this number may not be accurate.

Eleven lines with clear mc-GISH images were analysed and only 7 of the KASP™ marker results loosely corresponded (see Table 6.7). Figure 6.11 shows one such line and displays how much work is needed to improve the accuracy of the KASP™ markers.

In a separate experiment, a different 89 lines with clear sc-GISH images available were analysed via PCR with a selected 84 KASP markers that covered the whole wheat genome. Out of the 89 lines analysed, only 11 matched their corresponding GISH results. This again suggests that whilst the KASP™ markers are capable of detecting the presence of *Th. elongatum* 401007 chromatin they cannot currently be used to identify or characterise segments due to reliability issues.

Research Overview and Wider Applications

In summary, the Affymetrix SNP markers are currently the most accurate option for high-throughput identification and tracking of recombinants from *Th. elongatum* 401007 in a wheat background. The 263 Affymetrix SNP markers produced genotypes that corresponded to the GISH images from the same lines (when available) and identified 202 introgressions from 352 lines. In order to fully utilise the KASP™ markers produced in this research, future work will need to focus on increasing the number of lines available for analysis with single segments. This will help the detection of faulty or missing markers. Offspring produced from lines already analysed with the KASP™ markers could be tested with the subset already used to see if it is possible to link single segments with individual markers. Any advances with the KASP™ markers will be aided by improvements made in the KASP™ marker protocol by the King Research Group since the completion of this work.

Whilst a fairly low throughput procedure, the sc-GISH proved to be the most effective and reliable way to detect the presence of *Th. elongatum* 401007 genomic material in a wheat background. It also supported the development of the Affymetrix SNP markers and helped identify problematic areas within the KASP™ marker work.

The development of more back-crossed lines with single, smaller-sized segments enable the markers developed in this work to be utilised for breeding purposes. Lines with large segments will potentially contain genes for multiple traits of interest; therefore, it would be very difficult to assign specific Affymetrix or KASP™ markers to any particular trait. The smaller the

segment, the fewer markers that will align and the simpler it will be for breeders to identify and track desired traits in back-crossing programmes. This would also help reduce the impact of linkage drag in any lines investigated for beneficial traits in field trials.

Regardless of the ongoing molecular marker work, this research supports the use of *Th. elongatum* 401007 for breeding with wheat to produce hybrid lines with increased genetic variation. As far as is known, this research was the first attempt to capture the full range of genetic variation from the *Th. elongatum* 401007 genome. It is not known currently whether the full range of genomic variation from *Th. elongatum* 401007 was transferred to wheat from this approach; however the number of introgressions is a promising result.

The introgressions transferred into a wheat background within this work could contain the genes for increased disease tolerance, increased drought resistance or increased photosynthetic capacity already reported within *Th. elongatum* (Reynolds *et al.*, 2001; Reynolds, Dreccer and Trethowan, 2007; Fu *et al.*, 2012). The sheer number of introgressions transferred, within a relatively short period of time, supports the use of *Ph1* mutants to enable homoeologous recombination to incorporate genetic variation from wild relatives of wheat into modern hexaploid bread wheat. Future work completed by breeders will theoretically enable the identification of beneficial and non-beneficial traits in back-crossed lines, which in turn can be further bred to either spread, or remove, such traits. It is important that the material generated from this breeding programme should be handled by experienced pre-breeders with experience in handling the breeding of multiple traits who will look past any yield penalties. The presence of recessive traits must be investigated too. Experiments completed on introgression lines in tomato using the wild relative *Lycopersicon pimpinellifolium* discovered QTLs for traits very important in processing tomato production including fruit size, shape, colour and yield (Tanksley *et al.*, 1996; Tanksley and McCouch, 1997). Therefore, a wild relative should not be ignored if it does not express traits that appear beneficial in its phenotype because it may contain a plethora of recessive genes that will be beneficial in elite cultivars (see Figure 7.3).

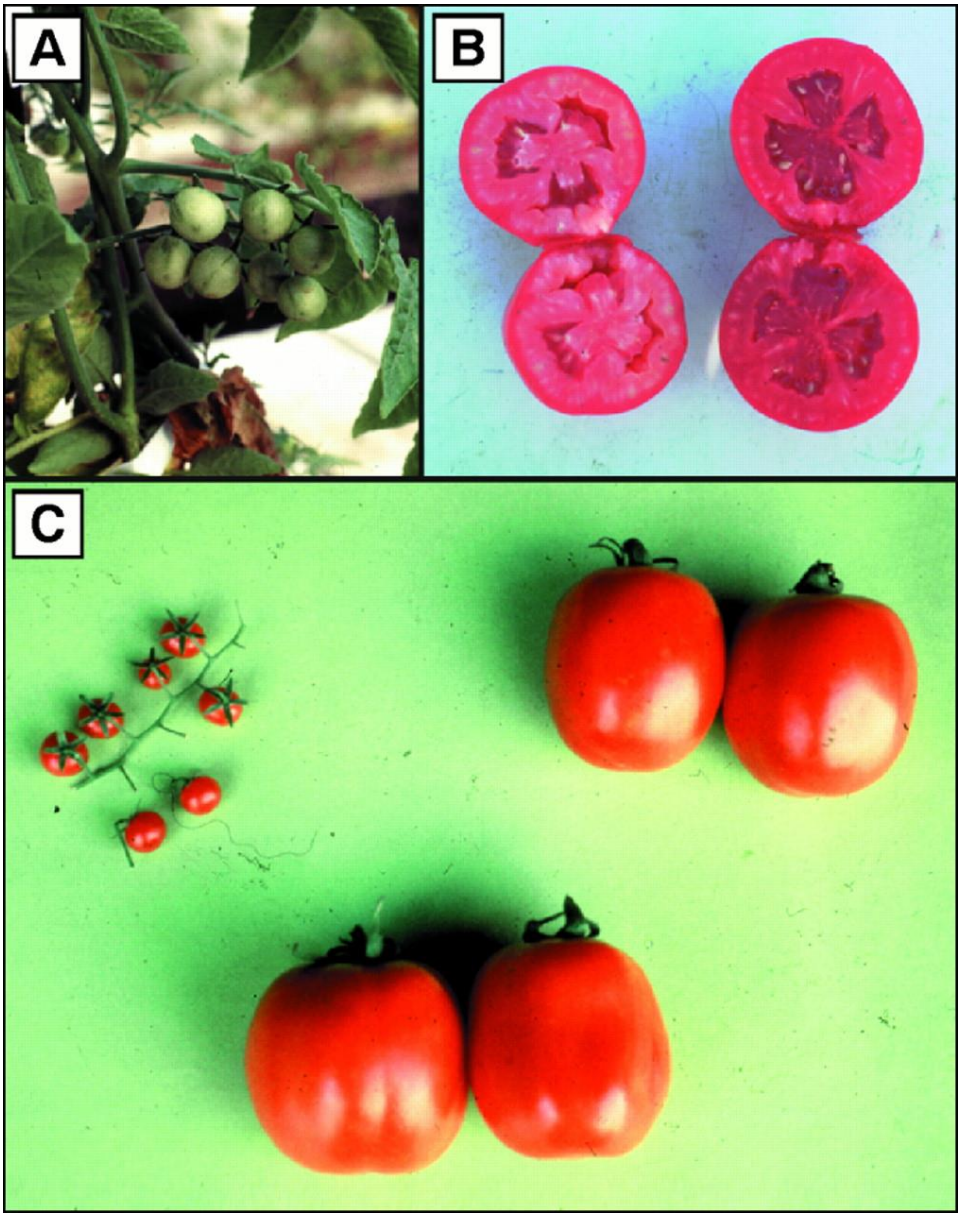


Figure 7.3: Image and text taken from Tanksley and McCouch (1997). (A) Wild tomato species *L. hirsutum* from Peru that produces small, inedible fruit that does not turn red upon ripening. (B) (Left) Fruit from modern processing tomato cultivar E6203. (Right) Fruit from nearly isogenic line (NIL) into which QTL for increased red pigment has been transferred from *L. hirsutum* by the advanced backcross QTL method (17). (C) (Top left) Fruit from *L. pimpinellifolium* from Peru that produces small berries typ- ical of most fruit-bearing wild species. (Top right) Fruit from modern processing tomato cultivar E6203. (Bottom center) Fruit from NIL into which QTL for increased fruit size has been transferred from *L. pimpinellifolium* by the advanced back- cross QTL method. Fruit of this NIL are significantly larger (10%) than the original E6203 variety.

The germplasm created in this work is not directly focussed on increasing yield, which is the focus of breeding companies, and therefore the material

needs analysis from a different view point that takes into account the value of variation within the gene pool of wheat for the future.

Backcrossing is a long process in general, however with *Th. elongatum* 401007 that process is slightly extended due to its slow growth habit and the maintenance of large numbers of *Th. elongatum* segments in late back-cross stages (BC_4 etc.). A large amount of work and patience will be required to advance to individuals with reduced numbers of segments and with reduced segment size. Lines with large segments could still be screened for traits of interest to reduce the overall number of plants taken forwards in the breeding programme. Each line would need to be screened for multiple traits, especially if they contain multiple segments in order to assess the maximum genetic variation contained within the lines.

The amount of variation available in the *Th. elongatum* genome could enable the production of wheat accessions able to withstand the impeding impacts of climate change, and to keep up with the increasing demand for the growing human population. This research also supports the use of the whole genome shotgun approach to generate back-crossed lines as opposed to screening lines for a particular trait of interest, as it enables a wider range of variation to be captured within a wheat background. It could also be argued that this approach is less wasteful, as breeding programmes focussing on a particular trait will use a large amount of resources to capture variation for one trait, whereas this programme captures variation for a plethora of traits simultaneously. The decreased cost and increased availability of markers available now in comparison to when this work first began may have enabled improved molecular marker results, however time constraints have limited this work so far. The decreased costs could also now enable the sequencing of *Th. elongatum* 401007 used to produce back-cross lines in this research in order to further understand its genomic composition and synteny with the wheat genome.

Concluding Remarks

In conclusion, at least 63,000 seed have been produced at various back-cross stages between *Th. elongatum* 401007 and wheat. Using GISH, 143 introgressions and 660 whole chromosomes from *Th. elongatum* 401007 have been identified in a wheat background. From the 263 Affymetrix SNP markers another 202 introgressions have been detected. The Affymetrix SNP

markers have also shown the conserved macro-synteny and collinearity between the genomes of wheat and *Th. elongatum* 401007, with the exception of chromosome 7E which is inverted in comparison to wheat. The Affymetrix SNP markers also enabled the production of a putative genetic linkage map for the genome of *Th. elongatum* 401007. The development of germplasm with a reduced number of segments and with reduced segment size would be highly beneficial for the advancement of the molecular markers. Until the marker systems are fully validated, the sc-GISH approach is a reliable and effective way to identify the genomic material from *Th. elongatum* 401007. The KASP™ markers will be an excellent future tool to identify and characterise introgressions from *Th. elongatum* 401007. The main advantage over the Affymetrix SNP markers will be the KASP™ markers ability to differentiate between heterozygous and homozygous introgressions. Homozygous segments are more desirable for future breeding efforts as they show greater genomic stability in progeny. Until improvements in the KASP™ markers are made, the Affymetrix SNP markers will be a powerful tool for detecting, characterising and tracking introgressions from *Th. elongatum* 401007 in a wheat background. All lines produced from this breeding programme have the potential to impact the future of wheat breeding by introducing new genetic variation for breeders to utilise in their efforts to increase wheat yields for future generations.

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9. Appendices

9.1. Buffers and Solutions protocol

3M Sodium Acetate Solution

To make 100ml of NaOAc (pH5.2):

1. Dissolve 40.8g of powdered NaOAc in 70ml of deionised water.
2. Adjust the pH to 5.2 by adding glacial acetic acid.
3. Add water to bring the total volume of the solution to 100ml.
4. Check pH once more with a pH meter.

Store at room temperature indefinitely.

6M Ammonium acetate ($NH_4C_2H_3O_2$)

To make 50ml:

1. Dissolve 23.124g of 6M $NH_4C_2H_3O_2$ in 50ml of de-ionised water
2. Store at 4^oC

DNA extraction buffer

To make 100ml mix together:

- 10ml of 1.0M Tris-HCL (pH 7.5)
- 10ml of 0.5M EDTA (pH 8.0)
- 12.5ml of 10% SDS
- 67.5ml of de-ionised H_2O

90% Ethanol + 10% 3M NaOAc (pH 5.2)

To make 100ml:

1. Add 90ml of 100% Ethanol to 10ml of 3M Sodium Acetate.
2. Mix well.

Store at room temperature indefinitely.

SS DNA (140µl/ml)

To make 50ml:

1. Add 5ml of 50x TAE (pH 6.3) to 44.3ml of de-ionised water.
2. Add 0.7ml of 10mg/ml SS DNA.
3. Store at 4^oC

20x SSC

To make a litre of 20x SSC:

1. Weigh out 175.3g of NaCl and 88.2g of $Na_3C_6H_5O_7$ (Trisodium citrate).
2. Dissolve in 800ml of deionised water.
3. Adjust the pH to 7.0 using drops of hydrochloric acid.
4. Adjust the volume to 1 litre by adding additional deionised water.
5. Autoclave to sterilise.

2x SSC

To make 1 litre of 2x SSC:

1. Measure out 900ml of deionised water.
2. Add 100ml of 20x SSC.
3. Mix well.

10 x T.E.

To make a solution with a pH of 7.5:

1. Mix 100mM of Tris to 10mM of EDTA

1 x T.E.

To make 100ml:

1. Mix 90ml of de-ionised water with 10ml of 10x T.E.

10X PBS (phosphate buffered saline)

To make 1 litre dissolve the following compounds in 1 litre of de-ionised water:

1. NaCl 80g
2. KCl 2g
3. Na2HPO4 14.4g
4. KH2PO4 2.4g
5. dH2O 1L

Autoclave for 20 minutes.

1x TAE buffer

To make a solution with a pH of 8.6:

1. Mix 40mM of Tris base with 20mM of Acetate
2. Add 1mM EDTA

Store at room temperature.

Rox PACE mix

1. Aliquot Rox-free PACE mix (Applied Biosystems) from a bottle of 250 ml to 25ml portions in 50ml tubes.

2. Make 500µl of 1:10 dilution of the Rox stock (Applied Biosystems) by adding 50µ of Rox Stock to 450µl dH2O, mix well.

3. Add 75µl of 1:10 dilution of Rox to a 25ml aliquot of Rox-free PACE, mix thoroughly and label the tube clearly Rox added.

Store /Freeze all Rox-PACE mix at -20C.

9.2. List of plants sent for Affymetrix analysis

BC_1	BC_2	BC_3	BC_4	BC_5	Addition Lines
BC_1-60A	BC_2-220A	BC_3-142A	BC_4-87A	BC_5-26A	CS/1E-2
BC_1-60B	BC_2-220B	BC_3-142C	BC_4-87B	BC_5-26B	CS/2E-1
BC_1-60C	BC_2-220C	BC_3-142D	BC_4-87C	BC_5-27A	CS/2ES-1
BC_1-60D	BC_2-221A	BC_3-142E	BC_4-88A	BC_5-27B	CS/2EL-2
BC_1-60E	BC_2-221B	BC_3-142F	BC_4-88B		CS/3E-2
BC_1-60F	BC_2-221C	BC_3-144A	BC_4-88C		CS/3ES-2
BC_1-61A	BC_2-222A	BC_3-144B	BC_4-88D		CS/3EL-1
BC_1-61B	BC_2-223A	BC_3-144C	BC_4-88E		CS/4E-1
BC_1-61C	BC_2-223B	BC_3-144D	BC_4-89A		CS/4Eα-1
BC_1-61D	BC_2-223C	BC_3-144E	BC_4-89B		CS/5E-1
BC_1-62A	BC_2-224A	BC_3-144F	BC_4-89D		CS/5ES-1
BC_1-62B	BC_2-224B	BC_3-146A	BC_4-89E		CS/5EL-1
BC_1-62C	BC_2-224C	BC_3-146C	BC_4-90A		CS/6E-1
BC_1-62D	BC_2-225A	BC_3-146D	BC_4-90C		CS/6ES-1
BC_1-62E	BC_2-225B	BC_3-146E	BC_4-90D		CS/6EL-1
BC_1-62F	BC_2-225C	BC_3-146F	BC_4-90E		CS/7E-2
BC_1-62G	BC_2-226A	BC_3-147A	BC_4-90F		CS/7ES-1
BC_1-62H	BC_2-226B	BC_3-147B	BC_4-117A		CS/7EL-1
BC_1-62J	BC_2-227A	BC_3-148A	BC_4-117B		
BC_1-76A	BC_2-227C	BC_3-149A	BC_4-117C		
BC_1-76B	BC_2-228B	BC_3-150A	BC_4-117D		
BC_1-76D	BC_2-229A	BC_3-151A	BC_4-117E		
BC_1-76E	BC_2-397A	BC_3-151B	BC_4-118A		
BC_1-158A	BC_2-397B	BC_3-215A	BC_4-118B		
BC_1-158B	BC_2-398A	BC_3-216A	BC_4-118C		

BC_1-158C	BC_2-399A	BC_3-216B	BC_4-118E
BC_1-158D	BC_2-400A	BC_3-217A	BC_4-171A
BC_1-158E	BC_2-400B	BC_3-217B	BC_4-171B
BC_1-158F	BC_2-401A	BC_3-218A	BC_4-172A
BC_1-158G	BC_2-401B	BC_3-218B	BC_4-172B
BC_1-159A	BC_2-402A	BC_3-219A	BC_4-172C
BC_1-159B	BC_2-402B	BC_3-220A	BC_4-174A
BC_1-159C	BC_2-403A	BC_3-220B	BC_4-176A
BC_1-159D	BC_2-403B	BC_3-221A	BC_4-176B
BC_1-159E	BC_2-405A	BC_3-280B	BC_4-176C
BC_1-159F	BC_2-405B	BC_3-281A	BC_4-176D
BC_1-196A	BC_2-406A	BC_3-281B	BC_4-179A
BC_1-196B	BC_2-406B	BC_3-282A	BC_4-179B
BC_1-196C	BC_2-408A	BC_3-282B	BC_4-179C
BC_1-196D	BC_2-409A	BC_3-283A	BC_4-179D
BC_1-196E	BC_2-409B	BC_3-381A	BC_4-180A
BC_1-196F	BC_2-410A	BC_3-381B	BC_4-180B
BC_1-196G	BC_2-410B	BC_3-381C	BC_4-180C
BC_1-196H	BC_2-411A	BC_3-381D	BC_4-180D
BC_1-196I	BC_2-411B	BC_3-381E	BC_4-181A
BC_1-196J	BC_2-412A	BC_3-382A	BC_4-181B
BC_1-196K	BC_2-412B	BC_3-382B	BC_4-183A
BC_1-196L	BC_2-413A	BC_3-383A	BC_4-183B
BC_1-196M	BC_2-414A	BC_3-383B	BC_4-183C
BC_1-196P	BC_2-414B	BC_3-386A	BC_4-183D
BC_1-196Q	BC_2-415A	BC_3-386C	BC_4-184A
BC_1-244A	BC_2-416A	BC_3-386D	BC_4-184B
BC_1-244B	BC_2-416B	BC_3-387A	BC_4-184C
BC_1-244C	BC_2-417A	BC_3-388A	BC_4-184D

BC_1-453A	BC_2-419A	BC_3-389A	BC_4-185A
BC_1-453B	BC_2-420A	BC_3-389B	BC_4-185B
BC_1-453C	BC_2-420B	BC_3-389C	BC_4-186A
BC_1-454A	BC_2-421A	BC_3-390A	BC_4-186B
BC_1-454B	BC_2-421B	BC_3-390B	BC_4-187A
BC_1-454C	BC_2-422A	BC_3-390D	BC_4-187B
BC_1-455A	BC_2-422B	BC_3-390E	BC_4-188A
BC_1-455B	BC_2-423A	BC_3-390F	BC_4-188B
BC_1-455C	BC_2-423B	BC_3-390G	BC_4-189A
BC_1-456A	BC_2-424A	BC_3-391A	BC_4-189B
BC_1-456B	BC_2-424B	BC_3-391B	BC_4-189C
BC_1-456C	BC_2-425A	BC_3-392A	BC_4-191A
BC_1-457A	BC_2-425B	BC_3-392B	BC_4-191B
BC_1-457B	BC_2-426A	BC_3-393A	BC_4-191C
BC_1-457C	BC_2-427A	BC_3-393B	BC_4-192A
BC_1-458A	BC_2-428A	BC_3-393C	BC_4-192B
BC_1-458B	BC_2-429A	BC_3-393D	BC_4-194A
BC_1-458C	BC_2-430A	BC_3-393E	BC_4-195A
	BC_2-432A	BC_3-394A	BC_4-195B
	BC_2-433B	BC_3-394B	
	BC_2-434A	BC_3-395A	
	BC_2-435A	BC_3-396A	
	BC_2-436A		
	BC_2-437A		
	BC_2-438A		
	BC_2-439A		
	BC_2-439B		
	BC_2-440A		
	BC_2-441A		

BC_2-442A
BC_2-442B
BC_2-442C
BC_2-684A
BC_2-684B
BC_2-685A
BC_2-685B
BC_2-686A
BC_2-686B
BC_2-687A
BC_2-687B
BC_2-704A
BC_2-705A
BC_2-705B
BC_2-706A
BC_2-706B
BC_2-706C
BC_2-707A
BC_2-707B
BC_2-707C

9.3. Chinese Spring Addition Line IDs

WRC ID Code	Collection (idPlant)	Accession Name	Breeder	Origin Country
CS/1E-2	WPGS (id#31961)	Chinese Spring + 1E [*Th. elongatum*]	Dvorak. J	USA
CS/2E-1	WPGS (id#31962)	Chinese Spring + 2E [*Th. elongatum*]	Dvorak. J	USA
CS/2ES-1	WPGS (id#28557)	Chinese Spring + 2E S [*Th. elongatum*]	Dvorak. J	USA
CS/2EL-2	WPGS (id#28556)	Chinese Spring + 2E L [*Th. elongatum*]	Dvorak. J	USA
CS/3E-2	WPGS (id#28560)	Chinese Spring + 3E [*Th. elongatum*]	Dvorak. J	USA
CS/3ES-2	WPGS (id#28559)	Chinese Spring + 3E S [*Th. elongatum*]	Dvorak. J	USA
CS/3EL-1	WPGS (id#28558)	Chinese Spring + 3E L [*Th. elongatum*]	Dvorak. J	USA
CS/4E-1	WPGS (id#28562)	Chinese Spring + 4E [*Th. elongatum*]	Dvorak. J	USA
CS/4Eα-1	WPGS (id#28561)	Chinese Spring + 4E a [*Th. elongatum*]	Dvorak. J	USA
CS/5E-1	WPGS (id#28565)	Chinese Spring + 5E [*Th. elongatum*]	Dvorak. J	USA
CS/5ES-1	WPGS (id#28564)	Chinese Spring + 5E S [*Th. elongatum*]	Dvorak. J	USA
CS/5EL-1	WPGS (id#28563)	Chinese Spring + 5E L [*Th. elongatum*]	Dvorak. J	USA
CS/6E-1	WPGS (id#28568)	Chinese Spring + 6E [*Th. elongatum*]	Dvorak. J	USA
CS/6ES-1	WPGS (id#28567)	Chinese Spring + 6E S [*Th. elongatum*]	Dvorak. J	USA
CS/6EL-1	WPGS (id#28566)	Chinese Spring + 6E L [*Th. elongatum*]	Dvorak. J	USA
CS/7E-2	WPGS (id#28571)	Chinese Spring + 7E [*Th. elongatum*]	Dvorak. J	USA
CS/7ES-1	WPGS (id#28570)	Chinese Spring + 7E S [*Th. elongatum*]	Dvorak. J	USA
CS/7EL-1	WPGS (id#28569)	Chinese Spring + 7E L [*Th. elongatum*]	Dvorak. J	USA

9.4. Plants sent for KASP marker analysis at LGC

BC_1	BC_2	BC_2F_1	BC_2F_2	BC_3	$BC3F_1$	BC_4	Addition Lines
BC_1-60A	BC_2-220A	BC_2F_1-124D	BC_2F_2-475A	BC_3-142C	BC_3F_1-174B	BC_4-118E	CS/1E-2
BC_1-60D	BC_2-223A	BC_2F_1-125A	BC_2F_2-475B	BC_3-147B	BC_3F_1-174C	BC_4-179C	CS/2E-1
BC_1-196C	BC_2-225A	BC_2F_1-125B	BC_2F_2-475C	BC_3-149A	BC_3F_1-175A	BC_4-179D	CS/2ES-1
BC_1-196M	BC_2-227A	BC_2F_1-126A	BC_2F_2-476A	BC_3-151A	BC_3F_1-175B	BC_4-181B	CS/2EL-2
BC_1-196N	BC_2-229A	BC_2F_1-126B	BC_2F_2-477A	BC_3-282A	BC_3F_1-176C	BC_4-183A	CS/3E-2
BC_1-244A	BC_2-397A	BC_2F_1-126C	BC_2F_2-477B	BC_3-283A	BC_3F_1-176D	BC_4-191B	CS/3ES-2
BC_1-454B	BC_2-406A	BC_2F_1-126D	BC_2F_2-478B	BC_3-391A	BC_3F_1-177A	BC_4-89D	CS/3EL-1
BC_1-456C	BC_2-409A	BC_2F_1-126F	BC_2F_2-479A	BC_3-392A	BC_3F_1-177B	BC_4-90A	CS/4E-1
BC_1-458E	BC_2-430A	BC_2F_1-126G	BC_2F_2-479B	BC_3-393B	BC_3F_1-177C	BC_4-90E	CS/4Eα-1
	BC_2-432A	BC_2F_1-126H	BC_2F_2-479C	$BC_3$395A			CS/5E-1
	BC_2-437A	BC_2F_1-126I	BC_2F_2-480A				CS/5ES-1
	BC_2-686A	BC_2F_1-127B	BC_2F_2-480B				CS/5EL-1
		BC_2F_1-128A	BC_2F_2-480C				CS/6E-1
		BC_2F_1-129A	BC_2F_2-480D				CS/6ES-1
		BC_2F_1-	BC_2F_2-				CS/6EL-1

129B	481A	
BC_2F_1-129C	BC_2F_2-481B	CS/7E-2
BC_2F_1-129D	BC_2F_2-481C	CS/7ES-1
BC_2F_1-129E	BC_2F_2-481D	CS/7EL-1
BC_2F_1-130A	BC_2F_2-482A	
BC_2F_1-130B	BC_2F_2-482B	
BC_2F_1-130C	BC_2F_2-483A	
BC_2F_1-130D	BC_2F_2-483B	
BC_2F_1-130E	BC_2F_2-483C	
BC_2F_1-130F	BC_2F_2-485A	
BC_2F_1-130G	BC_2F_2-485D	
BC_2F_1-130H	BC_2F_2-486A	
BC_2F_1-131A	BC_2F_2-486B	
BC_2F_1-131B	BC_2F_2-486C	
BC_2F_1-131F	BC_2F_2-487A	
BC_2F_1-131G	BC_2F_2-488A	
BC_2F_1-132A	BC_2F_2-488B	
BC_2F_1-	BC_2F_2-	

132B	488C
BC_2F_1-	BC_2F_2-
132C	488D
BC_2F_1-	BC_2F_2-
132D	490A
BC_2F_1-	BC_2F_2-
132E	490B
BC_2F_1-	BC_2F_2-
132F	490C
BC_2F_1-	BC_2F_2-
133A	490D
BC_2F_1-	BC_2F_2-
133B	491A
BC_2F_1-	BC_2F_2-
133C	491C
BC_2F_1-	BC_2F_2-
133D	494A
BC_2F_1-	BC_2F_2-
133F	494B
BC_2F_1-	BC_2F_2-
133G	494C
BC_2F_1-	BC_2F_2-
134A	494D
BC_2F_1-	BC_2F_2-
134C	495C
BC_2F_1-	BC_2F_2-
134D	496A
BC_2F_1-	BC_2F_2-
134E	496B
BC_2F_1-	BC_2F_2-
134G	496D
BC_2F_1-	BC_2F_2-
135C	497A
BC_2F_1-	BC_2F_2-

135D	497B
BC_2F_1-135E	BC_2F_2-498A
BC_2F_1-135G	BC_2F_2-498B
BC_2F_1-136A	BC_2F_2-499B
BC_2F_1-136B	BC_2F_2-499C
BC_2F_1-136D	BC_2F_2-500A
BC_2F_1-136E	BC_2F_2-500B
BC_2F_1-136F	BC_2F_2-503A
BC_2F_1-136H	BC_2F_2-503B
BC_2F_1-136I	BC_2F_2-503C
BC_2F_1-136J	BC_2F_2-503D
BC_2F_1-137A	BC_2F_2-504A
BC_2F_1-138A	BC_2F_2-505A
BC_2F_1-138B	BC_2F_2-505B
BC_2F_1-139A	BC_2F_2-505D
BC_2F_1-141A	BC_2F_2-506A
BC_2F_1-141C	BC_2F_2-506B
BC_2F_1-	BC_2F_2-

141D	506C
BC_2F_1-141F	BC_2F_2-506D
BC_2F_1-141G	BC_2F_2-507A
BC_2F_1-142A	BC_2F_2-507B
BC_2F_1-142B	BC_2F_2-508B
BC_2F_1-142C	BC_2F_2-508C
BC_2F_1-142D	BC_2F_2-509A
BC_2F_1-142E	BC_2F_2-509B
BC_2F_1-142F	BC_2F_2-509C
BC_2F_1-142G	BC_2F_2-509D
BC_2F_1-142H	BC_2F_2-511A
BC_2F_1-142I	BC_2F_2-511B
BC_2F_1-143A	BC_2F_2-512B
BC_2F_1-143B	BC_2F_2-512C
BC_2F_1-143D	BC_2F_2-513A
BC_2F_1-144A	BC_2F_2-513B
BC_2F_1-145A	BC_2F_2-514A
BC_2F_1-	BC_2F_2-

145B	514C
BC_2F_1-146A	BC_2F_2-514D
BC_2F_1-146B	BC_2F_2-515A
BC_2F_1-146D	BC_2F_2-515B
BC_2F_1-146E	BC_2F_2-515C
BC_2F_1-147A	BC_2F_2-516A
BC_2F_1-147B	BC_2F_2-516B
BC_2F_1-147C	BC_2F_2-517A
BC_2F_1-147D	BC_2F_2-517B
BC_2F_1-147E	BC_2F_2-517C
BC_2F_1-147F	BC_2F_2-518A
BC_2F_1-147G	BC_2F_2-519A
BC_2F_1-147H	BC_2F_2-519B
BC_2F_1-148A	BC_2F_2-519C
BC_2F_1-148B	BC_2F_2-519D
BC_2F_1-148C	BC_2F_2-520A
BC_2F_1-148D	BC_2F_2-520B
BC_2F_1-	BC_2F_2-

148E	520C
BC_2F_1- 148F	BC_2F_2- 521A
BC_2F_1- 148G	BC_2F_2- 522A
BC_2F_1- 148H	BC_2F_2- 522C
BC_2F_1- 149A	BC_2F_2- 522D
BC_2F_1- 149B	BC_2F_2- 523A
BC_2F_1- 149C	BC_2F_2- 524A
BC_2F_1- 149D	BC_2F_2- 524B
BC_2F_1- 149E	BC_2F_2- 524C
BC_2F_1- 149F	BC_2F_2- 525A
BC_2F_1- 149G	BC_2F_2- 525B
BC_2F_1- 149H	BC_2F_2- 526A
BC_2F_1- 149J	BC_2F_2- 526B
BC_2F_1- 149K	BC_2F_2- 526C
BC_2F_1- 149L	BC_2F_2- 526D
BC_2F_1- 150A	BC_2F_2- 527A
BC_2F_1- 150B	BC_2F_2- 527C
BC_2F_1-	BC_2F_2-

150C	527D
BC_2F_1-150E	BC_2F_2-529A
BC_2F_1-151B	BC_2F_2-529B
BC_2F_1-151C	BC_2F_2-529C
BC_2F_1-151D	BC_2F_2-529D
BC_2F_1-152B	BC_2F_2-530A
BC_2F_1-152C	BC_2F_2-530B
BC_2F_1-153A	BC_2F_2-530C
BC_2F_1-153B	BC_2F_2-530D
BC_2F_1-153C	BC_2F_2-531A
BC_2F_1-153E	BC_2F_2-531C
BC_2F_1-153F	BC_2F_2-532A
BC_2F_1-153H	BC_2F_2-532B
BC_2F_1-153I	BC_2F_2-532C
BC_2F_1-154A	BC_2F_2-532D
BC_2F_1-154B	BC_2F_2-533A
BC_2F_1-154C	BC_2F_2-533B
BC_2F_1-	BC_2F_2-

154E	533C
BC_2F_1-155A	BC_2F_2-533D
BC_2F_1-155B	BC_2F_2-534A
BC_2F_1-156A	BC_2F_2-534B
BC_2F_1-156D	BC_2F_2-535A
BC_2F_1-156F	BC_2F_2-535B
BC_2F_1-156G	BC_2F_2-536B
BC_2F_1-156H	BC_2F_2-537A
BC_2F_1-157A	BC_2F_2-537B
BC_2F_1-157B	BC_2F_2-537C
BC_2F_1-157C	BC_2F_2-538A
BC_2F_1-157D	BC_2F_2-538B
BC_2F_1-157E	BC_2F_2-538C
BC_2F_1-157F	BC_2F_2-538D
BC_2F_1-157G	BC_2F_2-539A
BC_2F_1-157H	BC_2F_2-541A
BC_2F_1-157I	BC_2F_2-542A
BC_2F_1-	BC_2F_2-

157J	542B
BC_2F_1-	BC_2F_2-
158A	542C
BC_2F_1-	BC_2F_2-
158B	542D
BC_2F_1-	BC_2F_2-
159A	544A
BC_2F_1-	BC_2F_2-
160A	544B
BC_2F_1-	BC_2F_2-
160C	544C
BC_2F_1-	BC_2F_2-
161A	544D
BC_2F_1-	BC_2F_2-
161B	545A
BC_2F_1-	BC_2F_2-
161C	545B
BC_2F_1-	BC_2F_2-
161D	545C
BC_2F_1-	BC_2F_2-
161E	545D
BC_2F_1-	BC_2F_2-
162A	546A
BC_2F_1-	BC_2F_2-
162B	548A
BC_2F_1-	BC_2F_2-
162C	548B
BC_2F_1-	BC_2F_2-
162D	549A
BC_2F_1-	BC_2F_2-
162E	549B
BC_2F_1-	BC_2F_2-
162F	549C
BC_2F_1-	BC_2F_2-

162G	549D
BC_2F_1-162H	BC_2F_2-550A
BC_2F_1-162I	BC_2F_2-550C
BC_2F_1-163A	BC_2F_2-551A
BC_2F_1-163B	BC_2F_2-551B
BC_2F_1-163C	BC_2F_2-551C
BC_2F_1-163D	BC_2F_2-551D
BC_2F_1-163E	BC_2F_2-552B
BC_2F_1-163F	BC_2F_2-553A
BC_2F_1-163G	BC_2F_2-553B
BC_2F_1-163H	BC_2F_2-554A
BC_2F_1-163J	BC_2F_2-554C
BC_2F_1-163K	BC_2F_2-555A
BC_2F_1-164A	BC_2F_2-555B
BC_2F_1-164B	BC_2F_2-555C
BC_2F_1-164C	BC_2F_2-555D
BC_2F_1-164E	BC_2F_2-556A
BC_2F_1-	BC_2F_2-

164F	556B
BC_2F_1-166A	BC_2F_2-556C
BC_2F_1-166B	BC_2F_2-557A
BC_2F_1-166C	BC_2F_2-557B
BC_2F_1-166D	BC_2F_2-557C
BC_2F_1-167A	BC_2F_2-557D
	BC_2F_2-558A
	BC_2F_2-558B
	BC_2F_2-558C
	BC_2F_2-558D
	BC_2F_2-559A
	BC_2F_2-559B

9.5. PCR plate layout for KASP marker PCRs

Plate 1

	WRC 0002						WRC 0022						WRC 0033						WRC 0044					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	Parag on1	Parag on2	Parag on3	Th. Elong1	Th. Elong2	Th. Elong3	Parag on1	Parag on2	Parag on3	Th. Elong1	Th. Elong2	Th. Elong3	Parag on1	Parag on2	Parag on3	Th. Elong1	Th. Elong2	Th. Elong3	Parag on1	Parag on2	Parag on3	Th. Elong1	Th. Elong2	Th. Elong3
B	BC3-383A	BC3-388E	BC3-390D	BC3-664A	BC3-667B	BC3-667E	BC3-383A	BC3-388E	BC3-390D	BC3-664A	BC3-667B	BC3-667E	BC3-383A	BC3-388E	BC3-390D	BC3-664A	BC3-667B	BC3-667E	BC3-383A	BC3-388E	BC3-390D	BC3-664A	BC3-667B	BC3-667E
C	BC3-668B	BC3-669A	BC3-669B	BC3-677A	BC3-677B	BC3-677C	BC3-668B	BC3-669A	BC3-669B	BC3-677A	BC3-677B	BC3-677C	BC3-668B	BC3-669A	BC3-669B	BC3-677A	BC3-677B	BC3-677C	BC3-668B	BC3-669A	BC3-669B	BC3-677A	BC3-677B	BC3-677C
D	BC3 F1-224C	BC3 F1-224D	BC3 F1-227A	BC3 F1-227B	BC3 F1-229B	BC3 F1-230D	BC3 F1-224C	BC3 F1-224D	BC3 F1-227A	BC3 F1-227B	BC3 F1-229B	BC3 F1-230D	BC3 F1-224C	BC3 F1-224D	BC3 F1-227A	BC3 F1-227B	BC3 F1-229B	BC3 F1-230D	BC3 F1-224C	BC3 F1-224D	BC3 F1-227A	BC3 F1-227B	BC3 F1-229B	BC3 F1-230D
E	BC3 F1-231B	BC3 F1-233B	BC3 F1-233C	BC3 F1-234B	BC3 F1-234C	BC4-172A	BC3 F1-231B	BC3 F1-233B	BC3 F1-233C	BC3 F1-234B	BC3 F1-234C	BC4-172A	BC3 F1-231B	BC3 F1-233B	BC3 F1-233C	BC3 F1-234B	BC3 F1-234C	BC4-172A	BC3 F1-231B	BC3 F1-233B	BC3 F1-233C	BC3 F1-234B	BC3 F1-234C	BC4-172A
F	BC4-172B	BC4-172C	BC4-176B	BC4-176C	BC4-179A	BC4-179C	BC4-172B	BC4-172C	BC4-176B	BC4-176C	BC4-179A	BC4-179C	BC4-172B	BC4-172C	BC4-176B	BC4-176C	BC4-179A	BC4-179C	BC4-172B	BC4-172C	BC4-176B	BC4-176C	BC4-179A	BC4-179C
G	BC4-179D	BC4-181B	BC4-183A	BC4-191A	BC4-239D	BC4-245A	BC4-179D	BC4-181B	BC4-183A	BC4-191A	BC4-239D	BC4-245A	BC4-179D	BC4-181B	BC4-183A	BC4-191A	BC4-239D	BC4-245A	BC4-179D	BC4-181B	BC4-183A	BC4-191A	BC4-239D	BC4-245A
H	BC4-245B	BC4-245C	BC4-249A	BC4-249B	BC4-250B	BC4-250C	BC4-245B	BC4-245C	BC4-249A	BC4-249B	BC4-250B	BC4-250C	BC4-245B	BC4-245C	BC4-249A	BC4-249B	BC4-250B	BC4-250C	BC4-245B	BC4-245C	BC4-249A	BC4-249B	BC4-250B	BC4-250C
I	BC4-89D	BC4 F1-100A	BC4 F1-101A	BC4 F1-101B	BC4 F1-101D	BC4 F1-103B	BC4-89D	BC4 F1-100A	BC4 F1-101A	BC4 F1-101B	BC4 F1-101D	BC4 F1-103B	BC4-89D	BC4 F1-100A	BC4 F1-101A	BC4 F1-101B	BC4 F1-101D	BC4 F1-103B	BC4-89D	BC4 F1-100A	BC4 F1-101A	BC4 F1-101B	BC4 F1-101D	BC4 F1-103B
J	BC4 F1-107C	BC4 F1-108B	BC4 F1-108C	BC4 F1-108D	BC4 F1-110A	BC4 F1-110C	BC4 F1-107C	BC4 F1-108B	BC4 F1-108C	BC4 F1-108D	BC4 F1-110A	BC4 F1-110C	BC4 F1-107C	BC4 F1-108B	BC4 F1-108C	BC4 F1-108D	BC4 F1-110A	BC4 F1-110C	BC4 F1-107C	BC4 F1-108B	BC4 F1-108C	BC4 F1-108D	BC4 F1-110A	BC4 F1-110C
K	BC4 F1-112A	BC4 F1-112B	BC4 F1-90A	BC4 F1-90B	BC4 F1-90C	BC4 F1-91B	BC4 F1-112A	BC4 F1-112B	BC4 F1-90A	BC4 F1-90B	BC4 F1-90C	BC4 F1-91B	BC4 F1-112A	BC4 F1-112B	BC4 F1-90A	BC4 F1-90B	BC4 F1-90C	BC4 F1-91B	BC4 F1-112A	BC4 F1-112B	BC4 F1-90A	BC4 F1-90B	BC4 F1-90C	BC4 F1-91B
L	BC4 F1-92B	BC4 F1-92C	BC4 F1-92D	BC4 F1-94D	BC4 F1-95D	BC4 F1-99B	BC4 F1-92B	BC4 F1-92C	BC4 F1-92D	BC4 F1-94D	BC4 F1-95D	BC4 F1-99B	BC4 F1-92B	BC4 F1-92C	BC4 F1-92D	BC4 F1-94D	BC4 F1-95D	BC4 F1-99B	BC4 F1-92B	BC4 F1-92C	BC4 F1-92D	BC4 F1-94D	BC4 F1-95D	BC4 F1-99B
M	BC5-69B	BC5-73C	BC5-74C	BC5-75A	BC5-76A	BC5-76B	BC5-69B	BC5-73C	BC5-74C	BC5-75A	BC5-76A	BC5-76B	BC5-69B	BC5-73C	BC5-74C	BC5-75A	BC5-76A	BC5-76B	BC5-69B	BC5-73C	BC5-74C	BC5-75A	BC5-76A	BC5-76B
N	BC5-79B	BC5-83A	BC5-83B	BC5-87A	BC5-87B	BC5-87C	BC5-79B	BC5-83A	BC5-83B	BC5-87A	BC5-87B	BC5-87C	BC5-79B	BC5-83A	BC5-83B	BC5-87A	BC5-87B	BC5-87C	BC5-79B	BC5-83A	BC5-83B	BC5-87A	BC5-87B	BC5-87C
O	BC5-88B	BC5-88C	BC5-88D	BC5-90C	BC5-90D	BC5-91D	BC5-88B	BC5-88C	BC5-88D	BC5-90C	BC5-90D	BC5-91D	BC5-88B	BC5-88C	BC5-88D	BC5-90C	BC5-90D	BC5-91D	BC5-88B	BC5-88C	BC5-88D	BC5-90C	BC5-90D	BC5-91D
P	BC5-93A	BC5-93C	BC5-94A	BC5 F1-3A	BC5 F1-4A	blank	BC5-93A	BC5-93C	BC5-94A	BC5 F1-3A	BC5 F1-4A	blank	BC5-93A	BC5-93C	BC5-94A	BC5 F1-3A	BC5 F1-4A	blank	BC5-93A	BC5-93C	BC5-94A	BC5 F1-3A	BC5 F1-4A	blank

9.6. BLAST hits for Affymetrix SNP markers

Cells highlight yellow indicate the BBH for that marker.

#	Marker ID	cM	Elong Chr	Wheat Chr	A BLAST pos	Wheat Chr	B BLAST pos	Wheat Chr	C BLAST pos
1	AX-94647338	0	1E	1A	1210273	1B	1419791	1D	2147569
2	AX-94605037	0	1E			1B	8271108	1D	6265880
3	AX-94714460	1.749	1E	1A	7480848			1D	6399538
4	AX-94694937	2.909	1E			1B	8772906	1D	6476995
5	AX-94923857	3.485	1E	1A	11691168	1B	15142424		
6	AX-95091568	5.532	1E	1A	26554647	1B	40999694	1D	25731691
7	AX-95248435	7.282	1E	1A	30845354			1D	32740064
8	AX-94706334	7.282	1E	1A	32485071	1B	52760447	1D	34619013
9	AX-94600202	7.282	1E	1A	48692194	1B	69605659	1D	49588712
10	AX-94825050	7.282	1E	1A	52781502	1B	87458634	1D	53380946
11	AX-94431524	7.282	1E	1A	53595738	1B	90425378	1D	55411516
12	AX-94668789	7.282	1E	1A	58527735	1B	96241391	1D	60287855
13	AX-94659645	7.282	1E	1A	60978182	1B	100374718	1D	63020896
14	AX-94559449	7.282	1E	1A	67967070	1B	110751408	1D	69179557
15	AX-94838542	7.282	1E	1A	98610732	1B	132271340	1D	80447489
16	AX-94639519	7.282	1E			1B	156995645	1D	77010121
17	AX-95244854	7.282	1E	1A	110725875	1B	158255152	1D	108109856
18	AX-95088840	7.282	1E	1A	114137804	1B	167433722	1D	110334716
19	AX-94969312	7.282	1E	1A	126668338	1B	177548863	1D	116003162
20	AX-95181310	7.282	1E	1A	156905284	1B	206105620	1D	131290910
21	AX-94603132	10.233	1E	1A	226852803	1B	249093668		
22	AX-94609516	10.233	1E	1A	257169006	1B	296685411	1D	203318416
23	AX-95243397	11.1	1E	1A	258669282	1B	299229609	1D	204056097
24	AX-94415591	11.967	1E	1A	260430639	1B	299901346	1D	204478355

25	AX-94816469	11.967	1E	1A	260731103	1B	300148101	1D	204633724
26	AX-94614192	11.967	1E	1A	363138230	1B	320495507	1D	250746175
27	AX-94585755	11.967	1E	1A	304598636	1B	333169017	1D	240592901
28	AX-95247252	11.967	1E	1A	325809094	1B	357534128	1D	254610784
29	AX-94731169	11.967	1E	1A	319818263	1B	363932366	1D	250140005
30	AX-95249741	12.834	1E	1A	362460722	1B	391076809	1D	290447089
31	AX-94457686	13.701	1E	1A	365087871	1B	394002829	1D	293459188
32	AX-95188674	14.277	1E	1A	369692522	1B	399797117	1D	297526295
33	AX-94631754	15.437	1E	1A	376071765	1B	406785441	1D	301624918
34	AX-94614812	15.437	1E	1A	376887720	1B	408740709	1D	302378115
35	AX-94700816	15.437	1E	1A	376886974	1B	408741455	1D	302378861
36	AX-94815097	15.437	1E	1A	376676515	1B	409115644	1D	302708755
37	AX-95248891	15.437	1E	1A	398824941	1B	429224799	1D	317995967
38	AX-94959080	15.437	1E	1A	406178144	1B	434820942	1D	322381706
39	AX-95215437	16.304	1E	1A	408083406	1B	441526965	1D	327930543
40	AX-94680423	17.463	1E	1A	425310094			1D	328879677
41	AX-94976931	17.463	1E	1A	429831685	1B	446788614	1D	332575456
42	AX-94799746	17.463	1E	1A	432366172	1B	450542432	1D	335272744
43	AX-94619761	17.463	1E	1A	442794282	1B	460764002	1D	343889191
44	AX-94778702	17.463	1E	1A	462030245	1B	485975385	1D	363280574
45	AX-95201195	18.04	1E	1A	463422741	1B	487643693	1D	364770883
46	AX-94449858	18.907	1E			1B	488178189	1D	365298603
47	AX-94620619	18.907	1E	1A	463844802	1B	488248170	1D	365436461
48	AX-94879529	18.907	1E	1A	466522659	1B	491419715	1D	367429627
49	AX-94648380	18.907	1E			1B	510417130		
50	AX-94590777	19.194	1E	1A	499574210	1B	543565949	1D	404490127
51	AX-94923890	19.194	1E	1A	500159956	1B	544434677	1D	405112632
52	AX-94830058	19.194	1E	1A	502965851	1B	547971666	1D	407873726
53	AX-94811680	20.353	1E	1A	502966099	1B	547971913	1D	407873974

54	AX-94400160	20.353	1E	1A	509968062	1B	557357542	1D	413415744
55	AX-94889522	20.353	1E	1A	514286748	1B	564312270	1D	418045571
56	AX-94777625	20.93	1E	1A	517010379	1B	569679627	1D	421037416
57	AX-94927609	20.93	1E	1A	517614136	1B	570463526	1D	422056065
58	AX-94426657	20.93	1E	1A	520918061	1B	575270950	1D	426181095
59	AX-94984326	20.93	1E	1A	521239246	1B	575622537	1D	426599424
60	AX-95199064	21.217	1E	1A	539561033	1B	603710539	1D	444336901
61	AX-95239366	21.217	1E	1A	539749370	1B	604411040	1D	444865694
62	AX-94434557	21.217	1E	1A	545813011	1B	620668263	1D	451627063
63	AX-94696169	21.505	1E	1A	551236186	1B	631563476	1D	460159035
64	AX-94382911	21.505	1E	1A	560930651	1B	646634917	1D	468467843
65	AX-94758797	21.505	1E	1A	563697182	1B	652599796	1D	470482367
66	AX-95145266	22.081	1E	1A	574805966	1B	666472243	1D	478970113
67	AX-94764182	22.081	1E	1A	577186739	1B	668695987	1D	481075003
68	AX-94438392	22.081	1E	1A	587896667	1B	681199649	1D	488972853
69	AX-94675785	0	2E	2A	16086112	2B	24902468		
70	AX-94424382	0	2E	2A	16523810				
71	AX-94947404	5.764	2E	2A	18595090	2B	29556642	2D	17605335
72	AX-94943668	6.923	2E	2A	24135836			2D	22541774
73	AX-95191713	6.923	2E	2A	42476155	2B	65375492	2D	37613883
74	AX-94903195	6.923	2E	2A	42720363	2B	66215037	2D	37828636
75	AX-95256835	8.083	2E			2B	74001772	2D	46581121
76	AX-94681135	8.659	2E	2A	50959627	2B	75616460	2D	47422524
77	AX-94663017	8.947	2E	2A	62369345	2B	97394507	2D	62031868
78	AX-95252382	11.594	2E			2B	105992938	2D	69536387
79	AX-95113524	12.754	2E	2A	102686603	2B	154986086	2D	106321792
80	AX-95222525	12.754	2E			2B	166858639		
81	AX-94735763	12.754	2E	2A	119088248	2B	168622110	2D	117840249
82	AX-95172933	12.754	2E	2A	176892574	2B	225265109	2D	172992426

83	AX-94620995	12.754	2E	2A	209384458	2B	250728558	2D	194751887
84	AX-95175724	13.33	2E	2A	284608601				
85	AX-94944759	14.197	2E	2A	456495939	2B	410181825	2D	344969517
86	AX-95235860	14.485	2E	2A	530754282	2B	464681719	2D	392695843
87	AX-94896956	14.772	2E	2A	550627212	2B	482736359	2D	406955669
88	AX-94953488	15.348	2E	2A	574996961	2B	507828716	2D	430004718
89	AX-94885008	15.348	2E	2A	577308122	2B	510591634	2D	431680330
90	AX-95209735	15.348	2E	2A	582398277	2B	517436565	2D	439463097
91	AX-94793623	15.348	2E	2A	584512391	2B	518741377	2D	440768654
92	AX-95252073	15.636	2E	2A	584512592	2B	518741578	2D	440768855
93	AX-95185447	15.636	2E	2A	584512705	2B	518741691	2D	440768973
94	AX-94724796	15.923	2E	2A	590817254	2B	526670405	2D	446652662
95	AX-94893293	15.923	2E	2A	602118038	2B	539650983	2D	458720262
96	AX-94699666	15.923	2E	2A	615488238	2B	553876523	2D	473436854
97	AX-95092790	16.499	2E	2A	619859620	2B	559429751	2D	477435470
98	AX-94464916	16.787	2E	2A	631533800			2D	485987719
99	AX-94888719	17.074	2E	2A	631541058	2B	570898764	2D	485993834
100	AX-94891179	17.074	2E	2A	634105236	2B	574712815	2D	490570476
101	AX-95120881	17.074	2E	2A	637197415	2B	576696201	2D	491951990
102	AX-94863536	17.074	2E	2A	665708465	2B	612686744	2D	520524075
103	AX-94742957	17.074	2E	2A	671711934	2B	622922141	2D	526526324
104	AX-95127627	17.074	2E			2B	636091036	2D	534736147
105	AX-94567662	17.361	2E	2A	704758812	2B	675337578	2D	564077736
106	AX-94937792	17.361	2E	2A	706892535	2B	680044621	2D	567046192
107	AX-94939500	17.649	2E	2A	716456182	2B	695840381	2D	578928999
108	AX-94686979	18.225	2E	2A	738389432	2B	740313390	2D	606717489
109	AX-95630025	22.096	2E	2A	738389309	2B	740313513	2D	606717366
110	AX-94655425	28.507	2E			2B	743329373	2D	609244095
111	AX-94479185	30.257	2E	2A	744794363	2B	748432547	2D	613107237

112	AX-95129429	30.833	2E	2A	768627752	2B	793348942	2D	643776110
113	AX-95121028	0	3E	3A	23275445	3B	18784942	3D	13663694
114	AX-94839567	5.125	3E	3A	35050454	3B	41909723	3D	25976096
115	AX-89536025	5.412	3E	3A	61219798	3B	76407963	3D	48808264
116	AX-94468885	5.412	3E	3A	102273250	3B	133255193	3D	85250955
117	AX-94471678	5.412	3E	5B	211148109	3B	149835669		
118	AX-94809471	5.412	3E	3A	108376379	3B	162292144	3D	112690769
119	AX-94439463	5.7	3E	3A	112954004	3B	168257540	3D	116035670
120	AX-95173035	5.7	3E	3A	127970162	3B	171741648	3D	121533327
121	AX-94528741	5.7	3E	3A	159836014	3B	202188233	3D	141410294
122	AX-94604377	5.7	3E	7B	603948740	4B	428699960		
123	AX-95225885	7.449	3E	3A	428078701	3B	413597365	3D	317228537
124	AX-94439183	8.026	3E	3A	467153151	3B	446597858	3D	348165315
125	AX-94496167	8.026	3E	3A	477428563	3B	461463966	3D	358185046
126	AX-95199930	8.026	3E	3A	479100548	3B	464482431	3D	359638978
127	AX-94723713	8.026	3E			3B	486036357	3D	373609060
128	AX-94894258	8.026	3E	3A	538536933	3B	539236747	3D	414773702
129	AX-95091321	8.026	3E	3A	541967464	3B	544008627	3D	419523839
130	AX-95630395	8.026	3E			3B	577121912	3D	441199877
131	AX-94856472	8.026	3E	3A	585023099	3B	580470715	3D	444067165
132	AX-94931831	14.764	3E	3A	598563624	3B	600870593	3D	455886714
133	AX-95180897	14.764	3E			3B	620789922		
134	AX-94497957	14.764	3E	3A	610525422	3B	620790014	3D	468663280
135	AX-94877257	14.764	3E	3A	611702729	3B	621657243	3D	469162181
136	AX-94406001	14.764	3E	3A	616281010	3B	628805002	3D	473830108
137	AX-94598252	18.945	3E	3A	616288208	3B	628813209	3D	473837317
138	AX-95132136	19.232	3E	3A	631579378	3B	649309291	3D	487818076
139	AX-94741978	21.279	3E	3A	631584230	3B	649314382	3D	487822880
140	AX-94664855	21.279	3E	3A	638062444	3B	654367108	3D	496751915

141	AX-94874963	21.279	3E			3B	669035708	3D	508191324
142	AX-95260889	21.567	3E	3A	662395465	3B	696395050	3D	527673517
143	AX-94555409	21.567	3E	3A	674754383	3B	714385019	3D	539914485
144	AX-95012906	22.143	3E	3A	692477247	3B	736650521	3D	555150826
145	AX-94399214	24.49	3E			3B	780986653	3D	585923134
146	AX-94889002	37.881	3E	3A	749332842	3B	823378265	3D	613703568
147	AX-94794408	37.881	3E	3A	749332834	3B	823378273	3D	613703560
148	AX-94544839	0	4E	4A	595376411	4B	14340972	4D	7902539
149	AX-94513393	0.867	4E	4A	584124816	4B	28143983	4D	16246414
150	AX-94763454	2.027	4E	4A	584068062	4B	28192918	4D	16355938
151	AX-94531232	2.314	4E	2A	102327462	2B	154720797	2D	103166881
152	AX-95013746	2.314	4E	4A	450339958	4B	182239845	4D	119748632
153	AX-94629012	2.314	4E	4A	438617231			4D	128913370
154	AX-94698834	3.473	4E	4A	329182808	4B	358480571	4D	281298949
155	AX-94699337	3.761	4E	4A	182923401	4B	373762169	4D	300281422
156	AX-94468909	4.048	4E	4A	181621592	4B	374729541	4D	301021779
157	AX-94625842	4.624	4E	4A	175412184	4B	378767724	4D	303835881
158	AX-94618075	5.492	4E	4A	150229847	4B	398559401	4D	322674085
159	AX-94881176	6.068	4E	4A	150230370	4B	398559933	4D	322673558
160	AX-94661431	6.068	4E	4A	153252145	4B	400695469	4D	320037277
161	AX-94820364	6.068	4E	4A	136796386	4B	414798099	4D	336537822
162	AX-94563898	6.068	4E	4A	135615014	4B	416305627	4D	337152532
163	AX-94978002	9.939	4E	4A	132992597	4B	418520276	4D	339077665
164	AX-94696316	13.81	4E	4A	117280140	4B	428467206	4D	348310679
165	AX-95014532	14.969	4E	4A	116471600	4B	428970973	4D	348802338
166	AX-94472915	15.257	4E	4A	114587563	4B	433646453	4D	350501930
167	AX-94434996	17.304	4E	4A	103717374	4B	446515863	4D	361036550
168	AX-95108757	20.254	4E	4A	98597281	4B	450917795	4D	366326040
169	AX-94707138	20.254	4E	4A	87972681	4B	463717793	4D	377852800

170	AX-94443542	20.254	4E	4A	87412354	4B	464160632	4D	378262910
171	AX-95191433	21.708	4E	4A	59482630	4B	497552601	4D	402209210
172	AX-94758127	23.458	4E	4A	48531795	4B	511159432	4D	414995354
173	AX-94387796	26.713	4E	4A	16191593			4D	450509926
174	AX-94807329	26.713	4E	4A	11811427	4B	569630638	4D	455746040
175	AX-94705196	27.873	4E			4B	600922067	4D	476870248
176	AX-94503439	30.219	4E	4A	684661325	4B	604204008	4D	478033278
177	AX-94430472	30.795	4E	5A	664781708	4B	616274543	4D	484358277
178	AX-94681948	31.372	4E	5A	680209821			4D	499451971
179	AX-94925964	32.825	4E	5A	698359181	4B	657274162	4D	509480988
180	AX-94623475	0	5E			5B	16033800	5D	22441122
181	AX-94643290	0.287	5E	5A	79284406	5B	92233272		
182	AX-95214091	4.781	5E	5A	92174101	5B	103317935	5D	97037155
183	AX-94622909	7.128	5E	5A	173115023	5B	163089990	5D	184039993
184	AX-95166372	10.999	5E			5B	196269757	5D	193131241
185	AX-94983016	10.999	5E	5A	227707889	5B	209650704	5D	198952444
186	AX-94598591	11.286	5E	5A	307349385	5B	254573105	5D	228776744
187	AX-95157332	11.286	5E	5A	309100178	5B	255550390	5D	242966391
188	AX-94445676	13.633	5E					5D	313101557
189	AX-95244062	13.92	5E	5A	466986747	5B	432086465	5D	365325295
190	AX-94585720	14.787	5E	5A	485203212	5B	461137594	5D	384344057
191	AX-94451946	15.363	5E	5A	494870808	5B	471859840	5D	391737025
192	AX-95073230	21.45	5E			5B	539289191	5D	443008712
193	AX-94882660	22.026	5E	5A	591318697	5B	578367874	5D	471047408
194	AX-94852732	23.186	5E	5A	595037854	5B	582975465	5D	475575769
195	AX-94766275	23.186	5E	5A	598851795	5B	588500844	5D	480351898
196	AX-94409963	26.441	5E	5A	599218908			5D	480476392
197	AX-94579148	29.697	5E	4A	611196985				
198	AX-95165541	31.744	5E	4A	640003384	5B	671356286	5D	532247938

199	AX-95193570	0	6E					6D	3917370
200	AX-95191620	0	6E	6A	34982719	6B	64233881		
201	AX-95141129	4.182	6E	6A	79268695	6B	135859976	6D	62115302
202	AX-94784805	6.829	6E	6A	84922633	6B	142229560	6D	67377364
203	AX-86167869	6.829	6E	6A	90032550	6B	147961353	6D	73587857
204	AX-94555013	6.829	6E	6A	102106288	6B	161588687	6D	85040070
205	AX-95218218	6.829	6E	6A	104052449	6B	163969106	6D	86546850
206	AX-94888984	7.117	6E	6A	107113330	6B	169318510	6D	89004173
207	AX-95183306	7.117	6E	6A	110348253	6B	175726894	6D	91567850
208	AX-95143205	7.117	6E	6A	123536433	6B	188189414	6D	102467654
209	AX-94880647	7.117	6E	6A	135245170	6B	199763563	6D	110882126
210	AX-94932402	7.117	6E	6A	135247517	6B	199765840	6D	110884476
211	AX-94824033	7.404	6E	6A	137375714	6B	201408229	6D	112429665
212	AX-94721382	7.404	6E	5A	524244857	5B	496526921	5D	412365058
213	AX-94712565	8.271	6E	6A	171456070	6B	229724853	6D	132410006
214	AX-94876114	8.271	6E	6A	258141313	6B	309746964	6D	163836156
215	AX-94975749	8.558	6E	6A	356971915	6B	347226740	6D	262752104
216	AX-94666942	8.558	6E			6B	362872739	6D	189415635
217	AX-95193304	9.135	6E	6A	276310378			6D	206550098
218	AX-94748397	9.422	6E	6A	431175705	6B	453928382	6D	291960047
219	AX-94872125	9.998	6E	6A	435774967	6B	449767324	6D	289217470
220	AX-94536363	9.998	6E					6D	290319933
221	AX-95252343	9.998	6E	6A	421771087	6B	461263174	6D	296199685
222	AX-94407381	10.866	6E	6A	410893883	6B	470129793	6D	304288987
223	AX-94419941	10.866	6E	6A	409091513	6B	471230084	6D	305358871
224	AX-94946469	10.866	6E	6A	441499976	6B	473064761	6D	306137963
225	AX-94562108	10.866	6E	6A	454939689	6B	506723185	6D	317474276
226	AX-94541273	10.866	6E	6A	499645351	6B	539054761	6D	357567621
227	AX-94761183	10.866	6E	6A	503767256	6B	545219370	6D	363060458

228	AX-94664517	10.866	6E	6A	517664625	6B	562558813	6D	375811144
229	AX-94495972	10.866	6E	6A	522226196	6B	568256903	6D	380799454
230	AX-94956915	10.866	6E	6A	525993962	6B	574021945	6D	383746882
231	AX-94611381	11.153	6E	6A	528990120	6B	574627020	6D	384362351
232	AX-94797868	11.153	6E	6A	568494582	6B	639147632	6D	423788306
233	AX-95130177	11.729	6E	6A	573508327	6B	645549279	6D	428923479
234	AX-94532804	13.183	6E	6A	574479728	6B	646629800	6D	429311494
235	AX-95192178	14.932	6E	6A	585196836	6B	660676196	6D	436887495
236	AX-94768784	15.799	6E	6A	602706285	6B	693092765	6D	455886116
237	AX-95142148	15.799	6E	6A	605103946	6B	696993294	6D	458529647
238	AX-95072546	0	7E	7A	700637432	7B	699804384	7D	610780142
239	AX-94699229	0	7E	7A	644060822	7B	607605123	7D	559416280
240	AX-94938225	1.749	7E	7A	625566923			7D	543686062
241	AX-95081313	2.037	7E	7A	612039478	7B	572007391	7D	531576385
242	AX-94513629	2.037	7E			7B	565881249	7D	528089422
243	AX-94889553	2.324	7E	7A	596642613	7B	553598507	7D	520357632
244	AX-94468312	4.671	7E	7A	514936877	7B	505541545	7D	479697500
245	AX-94701218	4.671	7E	7A	514936944	7B	505541456	7D	479697411
246	AX-94539848	4.671	7E					7D	457971005
247	AX-94649688	4.958	7E	7A	501574700	7B	448702041	7D	431209283
248	AX-94712104	5.534	7E	7A	514597909	7B	434812371	7D	418918545
249	AX-95181221	6.111	7E			7B	388139516	7D	391532758
250	AX-95107279	6.111	7E					7D	388593269
251	AX-94487259	9.366	7E	7A	427745960	7B	374698680	7D	379525343
252	AX-94385484	12.622	7E	7A	355617090	7B	342102812	7D	301977434
253	AX-94801375	13.198	7E	7A	294339315	7B	248522263	7D	268501736
254	AX-94533947	13.198	7E	7A	288853936	7B	245043371	7D	264698879
255	AX-95237806	13.486	7E	7A	187032176	7B	152769519	7D	183743168
256	AX-95140865	13.486	7E			7B	152757313		

257	AX-94581390	13.486	7E	7A	182544195	7B	144634324	7D	178558924
258	AX-94500577	13.486	7E	7A	122460833	7B	79283844	7D	118108367
259	AX-94905259	13.486	7E	7A	92965486	7B	42333332	7D	90954559
260	AX-95018673	14.062	7E	7A	82142933	7B	26748291	7D	79490036
261	AX-94758772	15.516	7E	7A	58791942			7D	55029019
262	AX-94456809	15.803	7E	7A	42076676	4A	650044562	7D	42649821
263	AX-94900297	16.67	7E	7A	979619	4A	724790450	7D	13006579