The production and mapping of wheat-rye introgression lines exploiting *Ph1*

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Abstract

Wheat is a staple food source across the globe. To cope with the increasing problems of global food security, higher yielding, stress tolerant wheat cultivars are necessary. This thesis describes a method of introgressing rye chromatin into wheat to make beneficial rye traits available in wheat germplasm and thus to facilitate the use of rye traits in the breeding of elite wheat cultivars.

A non-specific 'shotgun' method of incorporating wheat chromatin has been used and has successfully introgressed the whole rye genome into a wheat background. The Pairing homologue one locus, *Ph1,* is known to control homologous pairing in wheat and thus deletion mutants of *Ph1* have been used to attempt to force recombination between wheat and rye. The amount of recombination between wheat and rye was lower than expected indicating a further barrier inhibiting recombination between wheat and rye.

The introgression lines produced have been genotyped using a combination of genomic *in situ* hybridisation (GISH) and two single nucleotides polymorphism (SNP) based methods, i.e. the Axiom® 35K SNP wild relative array and a selection of KASP markers). Genotyping has enabled the identification and tracking of introgressions through successive generations of a crossing programme and has shown a range of novel introgression lines covering the whole rye genome. Using the SNP genotyping it was possible to produce a bin map of rye and compare this map to wheat, which has confirmed several structural changes in rye in comparison to wheat.

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I would also like to express my deepest gratitude to my partner Alison Rowell for her immeasurable support.

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1 General Introduction

1.1 Introduction to food security

Food security and crop production is an increasingly important global concern. The term food security was first used in the 1970s and the concept has evolved with time. It took until the 1996 World Food Summit to define food security as, 'when all people, at all times, have physical and economic access to sufficient safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life' (FAO 1996). Food security is multifaceted and differs on local, national, and then a global scale. Superficially food security seems to be a matter of food supply and distribution; but is complicated by social, cultural, and economic pressures. The production of food that provides calories as well as adequate nutrients is vital.

The global population is predicted to be 9 billion by 2050, meaning that to maintain current levels of nutrition global food production needs to increase by a minimum of, 60% although increases above 60% will be needed to reduce the current levels of malnutrition (Thomson 2003; Freedman 2014). Increasing numbers of mouths to feed puts pressure on global agriculture to increase production. However, increases in production will not be easy to obtain compared to historical advances.

Increasing production is problematic due to:

- Climate change and the associated need to reduce agronomic inputs (such as fertilizer and fuel use)
- Limited land available for agricultural expansion
- Limited water supplies
- Increased competition from biofuels, housing, etc

1.2 Climate and Agriculture

Global climate change is intimately associated with agriculture. While climate change will alter agricultural practices, agriculture also has the potential to positively and negatively influence climate change. Climate change is affected by a large number of complex variables and is therefore difficult to model, which in turn has meant that predicting the effects of climate change are exceptionally difficult (Nelson *et al.* 2014).

Global agriculture needs to play a role in halting climate change. Agriculture and forestry are responsible for 21% of global greenhouse emissions, such as carbon dioxide, methane and nitrous oxide that cause the greenhouse effect heating the globe. For example, cattle and nitrogen based fertilisers are major contributors to emissions of methane and nitrous oxide respectively (FAO 2016). Reducing fertiliser input would therefore be beneficial.

Historically, increased food production has been attained through expanding the area of farmed land. However, the amount of land available to agriculture is finite and is under growing pressure from biofuels, housing, etc. Expansion into new arable land is not advisable, as it will have adverse effects on biodiversity, the climate, and is an expensive unsustainable option. Hence, there is growing pressure to reduce the amount of land used for agriculture. Reducing agricultural land enables the redevelopment of natural ecosystems increasing biodiversity and helping to store carbon. In order to make this reduction in agricultural land, yields need to increase (Lamb *et al.* 2016).

Climate change has both direct and indirect effects on plant growth. The indirect effects will essentially be a by-product of temperature rises. Equatorial regions will become hotter and are likely to become more arid, resulting in less amenable agricultural land, while northern regions, such as

the tundra, will have more useable land (Zhang and Cai 2011). A change to warmer wetter climates is likely to promote pathogen growth (Evans *et al.* 2008). The major direct effects of climate change will be due to increased CO_2 and increased temperature. Higher temperatures and changes in CO_2 will directly affect photosynthesis and will vary depending on the plant and environment (Kirschbaum 2004)

Increased climate variability and extremes as well as an increased pathogen load are predicted, meaning more biotic and abiotic stresses on crop plants. It is therefore vital to produce crops with higher levels of stress tolerance.

In summary the challenges for global agriculture are pressing and diverse. It is necessary to:

- Produce more, nutritious, food
- Use less land
- Use less agrochemicals
- Adapt to more biotic and abiotic stress

To produce more from less, the world needs to intensify agriculture in a sustainable way, termed sustainable intensification (Tilman *et al.* 2011) . To sustainably intensify agricultural production there are two key factors. Firstly, improving land management, such as exploiting crop rotations and no till practices (Pretty and Bharucha 2014; Franke *et al.* 2018). Secondly, improving the genetics of crops and animals to produce more from less with higher stress tolerances.

A focus on wheats' role in food security

Wheat is the world's most traded crop. Wheat, along with rice, provides globally the majority of dietary calories. Wheat makes up 45.1% of the cereals used for food with rice contributing 36.1% (FAO 2017). Wheat also provides most of the worlds cereal protein. Sugar cane, maize, wheat and

rice are the most productive crops (in weight of produce) with wheat being grown on the largest proportion of agricultural land. An estimated 760.1 million tonnes of wheat grain were produced in the 2016/2017 growing season with 754.79 million tonnes predicted for 2017/18. Data and predictions are from the FAOSTAT online resource, Agricultural market information system, AMIS http://statistics.amis-outlook.org/data/index.html accessed January 2018.

It is obvious that wheat is a major resource, and this plays a role in the global economy and food security. How wheat yields progress and how wheat adapts to climate change is vitally important. In the middle of the 20th century an improvement in wheat production averted a food crisis and was termed the green revolution. This was pioneered by Norman Borlaug through the development of high yielding dwarf varieties of wheat and their introduction to Mexico and Asia (Borlaug 1983; Hedden 2003). To cope with rising food demands and the changing climate a second green revolution is needed and can be achieved through the development of superior, higher-yielding lines of wheat. To obtain superior lines, modern technologies and breeding techniques need to be exploited.

1.3 Wheats History and Ancestry

To enhance wheat's genetics, it is necessary to understand its ancestry and evolutionary origins.

Triticeae

Wheat is a member of the tribe Triticeae. The Triticeae contains both diploid and polyploid species each with a basic haploid number of x=7, derived from a x=12 ancestor (Murat *et al.* 2014). The chromosomes of all Triticeae are large. Relationships between the members of the Triticeae are complicated because of the ease with which species hybridize, and the relative youth of the tribe. Ancestral hybridizations make it difficult to determine a species'

evolutionary history as the differences between genomes can become blurred (Feldman and Levy 2015). The Triticeae is an economically important tribe and as such a large amount of research has been undertaken to determine its evolutionary history. The tribe shows a highly reticulate evolution, due to multiple hybridisation events, resulting in a complicated phylogeny (Mason-Gamer 2004; Bernhardt *et al.* 2017). Reticulate evolution occurs when a new lineage has arisen through the merging of two ancestral linages, for example in a hybridisation event. Figure 1.1 shows the difference between a reticulate phylogenetic tree and the more standard bifurcating tree.

The genera *Aegilops, Amblyopyrum* and *Triticum* are known as the wheat group, as they are the genera containing species most closely related to wheat as well as wheat itself. The wheat group contains 13 diploid species that are divided into 7 groups based on the similarities between genomes, shown in table 1.1.

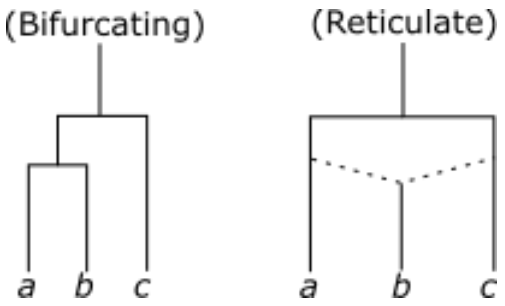


Figure 1.1. The left phylogenetic tree shows a standard phylogenetic tree with individual speciation events. The right phylogenetic tree shows a reticulate evolution, in which linage *b* is the result of a hybridisation between linages *a* and *c.*

Diploid species makeup the roots of the tribe but there are many allopolyploid and autopolyploid species, e.g. the non-wheat group Triticeae genus *Elymus* show the highest ploidy of 12x. Even diploid species in the Triticeae have been shaped by ancestral hybridisations. For example the D genome of *Aegilops tauschii* (2n = 2x =14, DD) is thought to have a

homoploid origin from an ancient hybridization between plants containing the A and the B genomes (Marcussen *et al.* 2014), although it should be noted that there is some debate as to whether the D genome was formed from a single ancestral hybridisation (Sandve *et al.* 2015) or a more complicated chain of events (Li *et al.* 2015b; Li *et al.* 2015c). The ease of the hybridisation in the Triticeae provides an opportunity to capture genes from related species.

Figure 1.2 shows a phylogenetic tree of the *Triticeae* produced by Petersen *et al*. (2006). For a more detailed phylogeny of the tribe see Bernhardt *et al.* (2017)

Table. 1.1. The diploid members of the wheat group (genera *Amblyopyrum, Aegilops* and *Triticum)* and their genomes.

Genus	Species	Genome
Amblyopyrum	*muticum*	T
Aegilops	*speltoides*	B (S)
	searsii	S
	bicornis	S
	longissima	S
	sharonensis	S
	tauschii	D
	caudata	C
	comosa	M
	uniaristata	M
	umbellulta	U
Triticum	*monococcum*	A
	urartu	A

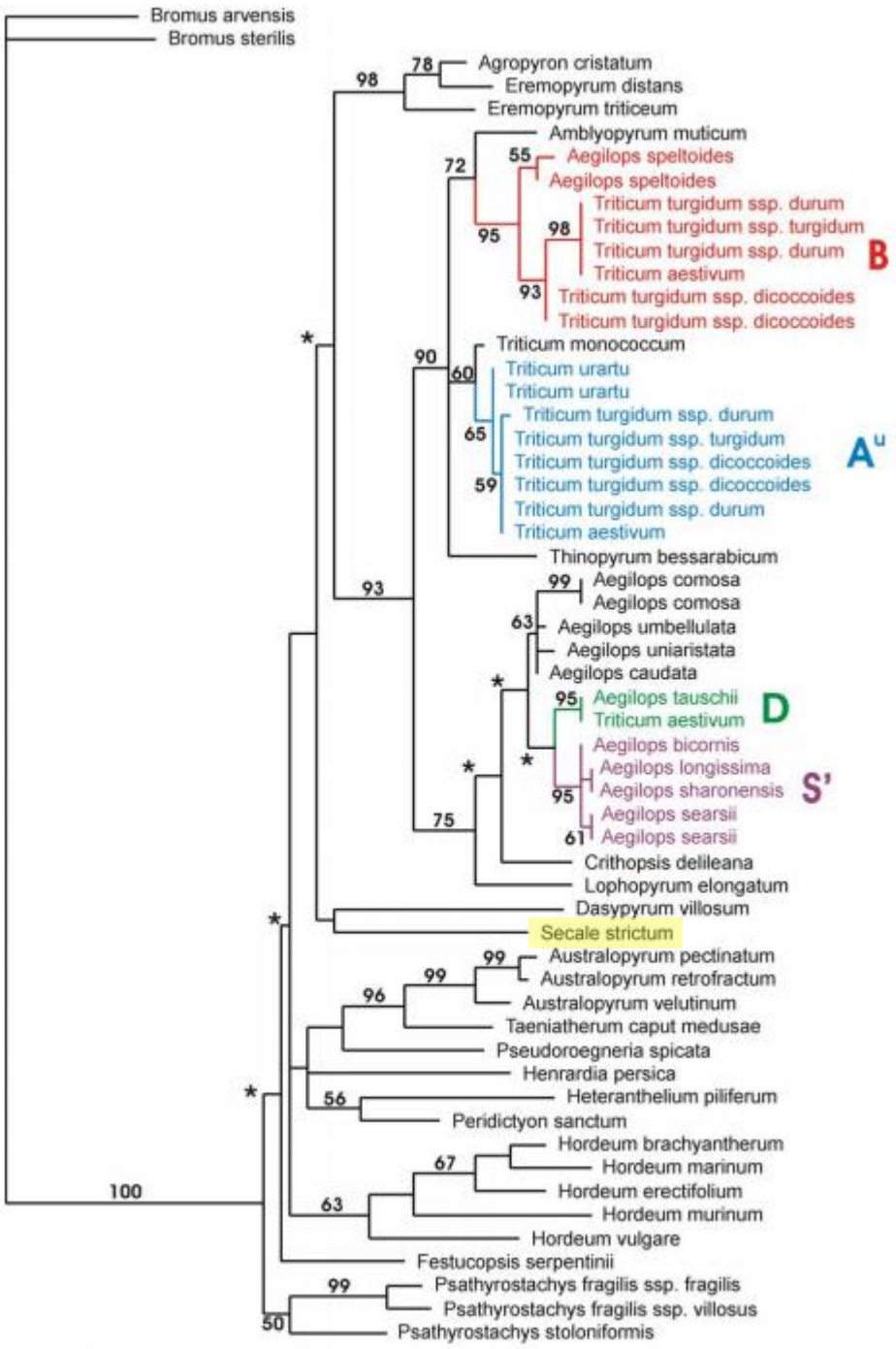


Figure. 1.2 The phylogenetic relationship of *Triticum and Aegilops* adapted from Petersen *et al*. (2006) The figure was derived from phylogenetic analysis of sequence data from the nuclear gene DMC1. Branches that collapse in the strict consensus tree are marked with an ¤. numbers above or below branches are jackknife proportions. It is important to note *Secale cereale* groups with *Secale strictum* (Highlighted in yellow) but is not shown. The groups containing the plausible progenitors of bread wheat are coloured.

1.4 *Wheat Evolutionary History and Bottlenecks*

Modern hexaploid bread wheat, *Triticum aestivum*, (2n = 6x = 42, AABBDD genomes) is an allohexaploid resulting from two hybridisations events demonstrated in figure 1.3. The first hybridisation event was between *Triticum urartu* (AA genome)(Dvorak *et al.* 1993) and a species closely related to *Aegilops speltiodes* (BB genome)(Dvorak and Zhang 1990) forming *Triticum turgidum* spp. *dicoccoides* (AABB genome). It is important to note when within wheat the *Ae. speltoides* like genome is designated as a B, but *Ae. speltoides* is usually designated as a S genome. The second hybridisation event was likely with a more domesticated subspecies of *Triticum turgidum, Triticum turgidum* spp. durum (AABB genome) and *Aegilops tauschii* (DD genome)(McFadden and Sears 1946) giving rise to *T. aestivum* (AABBDD).

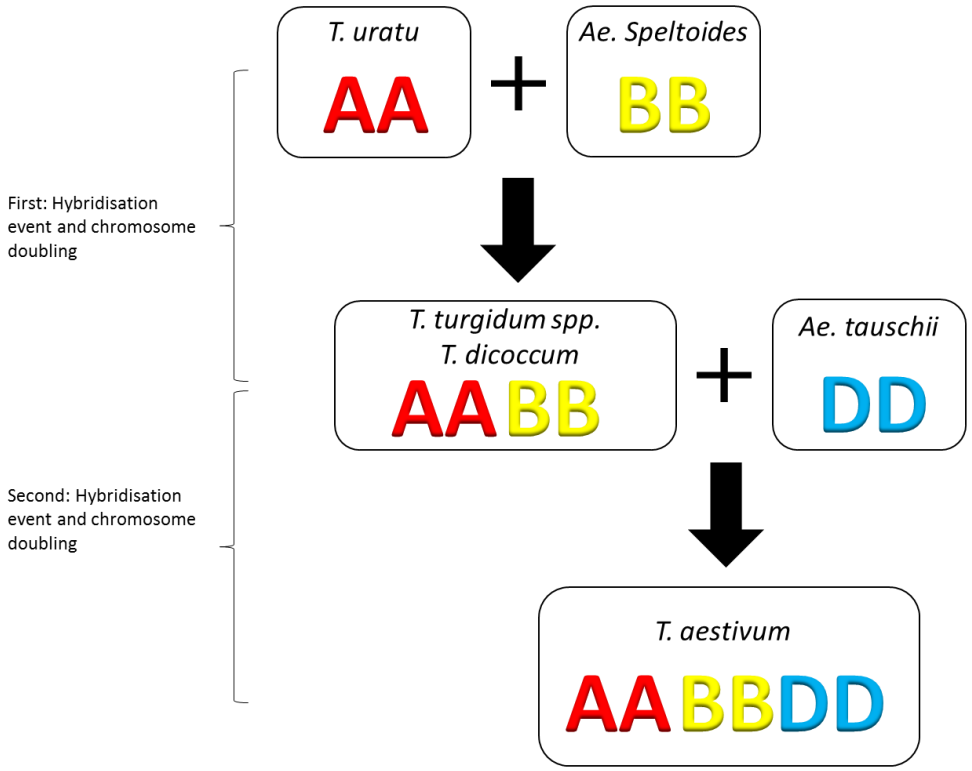


Figure 1.3 The hybridisation events that produced hexaploid bread wheat. *T aestivum.* Each hyrbidisation event consisted of a hybrid forming between the two parental plants and subsequent chromosome doubling

The hybridisation events isolated the neopolyploid from its' progenitors causing a genetic bottleneck, Figure 1.4, resulting in the neopolyploid species containing relatively little diversity. However, modern genotyping techniques have shown both bread and pasta wheat to be more diverse than previously estimated (K. Edwards, personal communication) , with most of the diversity due to introgressions from related species(White *et al.* 2008).

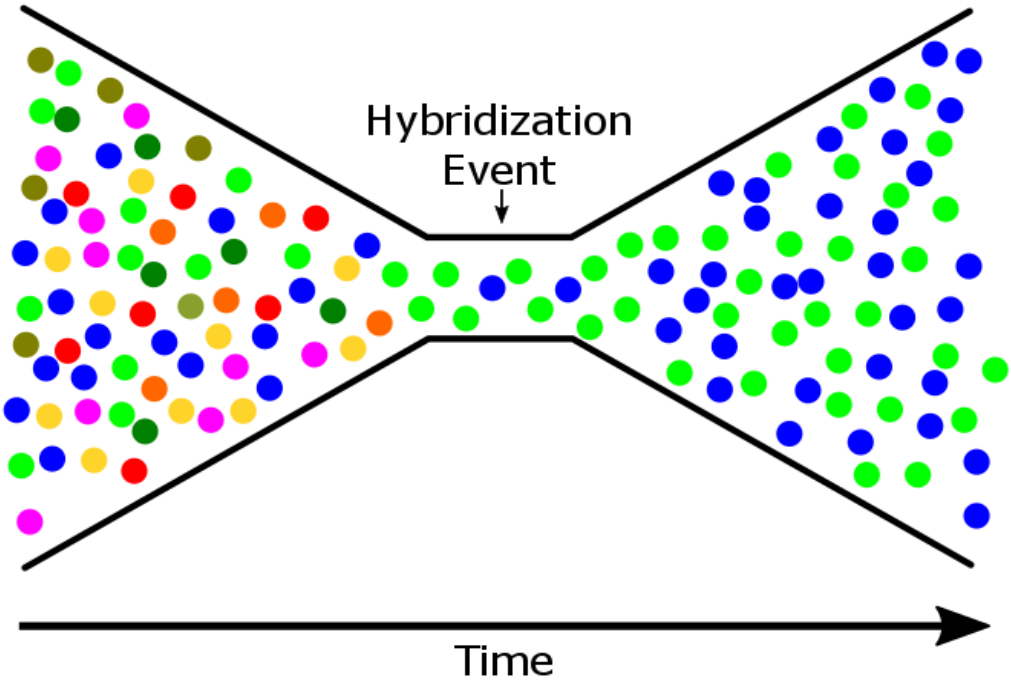


Figure 1.4. A representation of a genetic bottleneck, also known as the founder effect. Each circle is representative of a member of the population. Each colour represents different genotypes. Time progresses from left to right and is indicative of successive generations. The hybridization event isolates the neopolyploid, therefore later generations develop from one or a small number of individuals. The means genetic diversity is limited to what was present in the neopolyploid.

Wheat had a key role in human development. The domestication of wheat's ancestor, Emmer (*Triticum turgidum spp. dicoccoides*) 10,000 years ago, played a major role in humans moving from a hunter-gatherer lifestyle to an agrarian lifestyle (Tuberosa *et al.* 2014). Emmer and its descendants, including bread wheat (*T. aestivum*) and durum wheat (*Triticum turgidum spp. durum)* have been under selection pressure since domestication. Selection for important single genes such as *Br*, *Tg* and *Q*, which control brittle rachis, glume strength and free threshing respectively, are key for going from a wild to domesticated wheat (Feldman and Levy 2015). The section for these 'domestication' genes further reduces diversity, as alleles that were not present with these genes were unintentionally selected against.

Since the evolution of *T. aestivum*, its' genetic diversity has increased, mainly via natural mutations and introgressions from related species. For example, through most of its' history, *T. aestivum* has been grown in polygenic fields with related and wild species grown in close proximity. Polygenic fields have allowed the genome of wheat to broaden as geneflow between wheat and the nearby species was possible (Feldman and Levy 2015) this theory has recently been supported in by Ali *et al.* (2016) which showed the presence of novel rye and *Thinopyrum intermedium* regions in wheat breeding lines in the field . Hence for much of its' history, different regions world-wide and even individual farmers have selected for different traits, resulting in a diverse selection of wheat varieties, known as landraces.

It is generally, incorrectly, supposed that wheat diversity narrowed with the green revolution and the adoption of modern breeding practices, i.e. due to strong selection pressures, mono-crop fields and elite by elite breeding. However, the narrowing of elite germplasm has been more dependent on the growing region and breeding practices. For example, the USA has seen an increase in the level of diversity whereas Australia and the UK show a

more stable level of diversity (White *et al.* 2008). The diversity present within a crop species is the genetic basis on which the elite cultivars are produced. Elite cultivars themselves contain only a small proportion of the species gene pool. Theoretically, a perfect elite cultivar contains the best combination of genes from the whole gene pool. However, if a genepool does not contain the genetic diversity of a certain trait, it cannot be captured in the elite cultivar. For example, this might be particularly important if resistance is required to a particular disease. It is therefore beneficial to increase the total diversity of a crop species. The aim of increasing diversity is to also increase the number of beneficial and often rare gene alleles. The breeding of crop plants can be shown as a pyramid where the total genetic diversity in the species is shown by the base of the pyramid and elite cultivars are the peak, Figure 1.5.

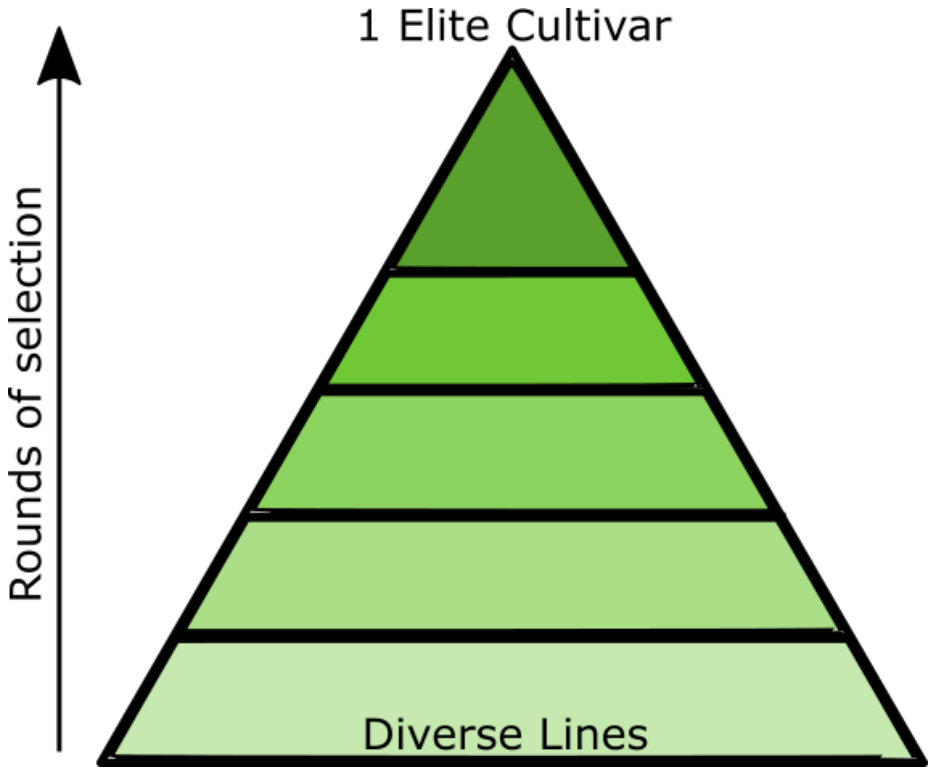


Figure 1.5. The breeding pyramids. The wider the bar the greater the diversity present. The vertical axis represents new generations and the rounds of selection. The darkening shades of green represent the concentration of desired genes. A single elite cultivar tops the pyramid.

1.5 Expanding Germplasm

The hexaploid nature of bread wheat means the genome has a large amount of redundancy and is amenable to the addition of genes from related sources. There are many ways of expanding wheats germplasm. Outlined below are three of the most promising methods.

- Landraces
- Synthetic wheat
- Wild relative introgression

Landraces

Individual farmers controlled agricultural practices before the green revolution. Farms in different locations are under differing selection pressures, due to diverse abiotic and biotic stresses as well as the aims of the farmer. A farmer would keep seed from year to year, obviously selecting seed from the best performing plants. This practice resulted in many different wheat varieties, containing adaptations dependent on their place of origin. Historic varieties collected from around the world are called landraces. These landraces contain useful diversity that can be exploited to improve modern elite varieties. Landraces can be crossed directly to elite cultivars meaning it is relatively simple to capture beneficial traits.

A. E. Watkins made one important collection in the 1930s. Originally more than 7,000 landraces were collected but unfortunately the majority were lost during the Second World War. The current collection contains 826 landraces from 32 countries. These cultivars are known as known as the Watkins landrace collection and are more diverse than current elite cultivars (Wingen *et al.* 2014). The Watkins collection has been shown to contain many resistance genes (Dyck 1994; Thompson and Seymour 2011; Bansal *et al.* 2013; Burt *et al.* 2014; Bansal *et al.* 2015; Li *et al.* 2016) but the search for more complicated traits has resulted in less success (Qamar *et al.* 2014).

Synthetics

The hybridisation events that created hexaploid wheat happened a modest number of times, most likely twice, and captured a random proportion of genes that are unlikely to contain all the possible beneficial alleles (Dvorak *et al.* 1998; Talbert *et al.* 1998; Charmet 2011). For example, *Ae. Tauschii* has been shown to be considerably more diverse than the D genome present within wheat (Lelley *et al.* 2000). Wheats' progenitor species contain variation for genes that would be beneficial in elite hexaploid wheat cultivars (van Ginkel and Ogbonnaya 2007).

To capture genes from hexaploid wheat progenitors it is possible to recreate the ancestral hybridisation events producing a synthetic wheat. *T. turgidum* (AABB genome) is crossed with *A. tauschii* (DD genome) and then the chromosomes are doubled either spontaneously or with colchicine treatment. The resulting plant is a new synthetic hexaploid wheat (AABBDD genome) (Sears 1939; Liu *et al.* 2002). The production of synthetics can be challenging as some accessions of *T. turdigum* and *Ae. Tauschii* appear to be incompatible with the chromosome doubling procedure resulting in synthetic wheats that lack chromosomes. Once produced, synthetic wheat can then be readily crossed to elite cultivars (Lange and Jochemsen 1992), allowing beneficial traits contained within *T. turgidum* or *Ae. tauschii* to be captured in elite cultivars.

Synthetic wheats have been extensively used as a source of beneficial genes. The international maize and wheat improvement centre is more commonly referred to as CIMMYT, (the acronym of its name in Spanish) have produced more than 1,000 synthetic wheat lines which have shown a wide range of traits associated with abiotic and biotic stress, quality and agronomic features, see table 1.2 adapted from van Ginkel and Ogbonnaya (2007).

Table. 1.2. The diverse traits shown in CIMMYT synthetics adapted from van Ginkel and Ogbonnaya (2007)

Trait	Reference
Agronomic features	
Yield (components): yield under irrigated conditions	Villareal *et al.* (1994c)
Yield under rainfed drought conditions	Reynolds *et al.* (1999)
High thousand kernel weight (up to 65 grams)	Calderini and Reynolds (2000)
High above ground biomass	Villareal *et al.* (1994c)
High photosynthetic rate	Del Blanco *et al* (2000)
Other morphological traits	Villareal *et al.* (1994b)
Abiotic tolerances	
Tolerance to drought	Reynolds *et al.* (1999)
Tolerance to frost at flowering	Maes *et a*l (2001))
Tolerance to salinity	Schachtman *et al.* (1992)
Tolerance to waterlogging	Villareal *et al.* (2001)
Biotic resistances	
Stripe rust (*Puccinia striiformis*)	Assefa and Fehrmann (2004)
Leaf rust (*Puccinia recondita*)	Ma *et al.* (1995)
Stem rust (*Puccinia graminis*)	Marais *et al.* (1994)
Leaf blotch (*Septoria tritici*)	Arraiano *et a*l (2001)
Glume blotch (*Septoria nodorum*)	Loughman *et al.* (2001))
Crown rot (*Fusarium graminearum*)	Mujeeb-Kazi *et al* . (2001b)
Yellow leaf spot (*Pyrenophora tritici-repentis*)	Cox *et al.* (1992)
Leaf blight (*Helminthosporium*)	Mujeeb-Kazi et al . (2001a)
Powdery mildew (*Erysiphe graminis*)	Kong *et al.* (1999)
Karnal bunt (*Neovossia indica*)	Villareal *et al.*(1994a)
Cereal cyst nematodes *(Heterodera avenae)*	Eastwood *et al.* (1991)
Root lesion nematodes *(Pratylenchus spp.)*	Thompson *et al.* (1999)
Greenbug (*Schizaphis graminum*)	Hollenhorst and Joppa (1983)
Hessian fly (*Mayetiola destructor*)	Tyler and Hatchett (1983)
Quality Features	
Proteins	William *et al.* (1993)
Glutenins	Pflünger *et al.* (2001)
Zinc efficiency	Cakmak *et al.* (1999)

Wild Relative Introgression

Transfer of genes from related species into wheat is known as wild relative introgression, although historically wild relative introgression was known as alien introgression and some work still uses the older term. Crossing wheat to related wild and cultivated species has occurred since breeding began with the first sterile interspecific wheat rye hybrids being reported in the late 19th century (Carman 1886; Roberts 1904). Wild relatives are members of the Triticeace with the potential to be gene donors for wheat. The work uses the term wild relative to include both wild and cultivated species, for example rye and barley (although crop plants themselves they are still classed as wild relatives). Capturing genes from wild relatives is challenging because of reproductive barriers between species, incompatibilities in pairing and recombination, and linkage drag. The strategy used to transfer genes from related species into wheat varies depending on the target donor species' evolutionary distance to wheat.

The potential of enriching wheat germplasm using wild relative introgression is vast. Much of the work undertaken on wild relatives has focused on disease resistance but there is potential for wild relatives to improve a much wider range of traits including quality and agronomic characteristics. (Reynolds *et al.* 2001; Monneveux *et al.* 2003; Wu *et al.* 2006; Garg *et al.* 2009; Ruiqi *et al.* 2014)

Capturing wild relative germplasm

The first method used to capture wild relative germplasm in wheat was the production of addition and substitution lines. Addition and substitutions lines contain complete chromosomes donated by a wild relative. Addition lines contain a complete set of wheat chromosomes and an additional pair of wild relative chromosomes (2n = 44) while substitution lines have one pair of wheat chromosomes replaced with a pair of chromosomes from a wild relative (2n = 42).

The second method is to produce wheat x wild relative introgression lines. Wheat wild relative introgression lines are wheat with a normal genome except one or more chromosome(s) contain a region of wild relative chromatin. The wild relative chromatin can be additive to the wheat chromosome or it may replace a region of chromatin.

There is some confusion in the terminology used when describing an introgression or a translocation. The term translocation is generally applied to the swapping of chromatin due to breakage and re-joining, e.g. in a Robertsonian translocation two chromosomes break at the centromere and re-join to the 'other' chromosome resulting in the transfer of a whole chromosome arm. An introgression is usually defined as when a segment of chromatin is transferred via recombination. In the transfer of a wild relative segment the recombination would be expected to occur between homoeologous chromosomes.

Wild Relative gene pools

Species within the Triticeace are related to wheat. The Triticeace contain a huge amount of diversity and are adapted to multiple environments. The Triticeace can be divided into three gene pools based on their evolutionary distance to wheat. The primary gene pool share their genome(s) with wheat, for example *Triticum monoccum* (AA) and *Triticum spelta* (AABBDD). The secondary gene pool shares a genome(s) with wheat as well as a more distantly related genome(s) for example *Triticum timopheevii* (AAGG) and *Triticum zhukovskyi* (GGAAAA). The tertiary gene pool contains species that are more distantly related to wheat and therefore do not contain homologous genomes, for example *Secale cereale* (RR) and *Thynopyrum intermedium* (JJEESS). Genes from the species in the primary gene pool can be captured by crossing and homologous recombination. Genes from species in the secondary gene pool can also be captured by crossing and homologous recombination, as long as the target gene in on the genome

shared by wheat (Friebe *et al.* 1996). Species from the tertiary gene pool are not homologous to wheat and therefore recombination between the donor species and wheat does not occur in a standard crossing scheme. Capturing genes from the tertiary gene pool into wheat requires more complicated strategies to be employed.

Techniques and challenges of Wild relative introgression

The simplest method of producing wild relative introgressions is to take the wild relative and cross directly to wheat. The wild relative x wheat produces an F_1 interspecific hybrid. The F_1 is then repeatedly back crossed to wheat and lines containing an introgression are selected. This simple method is limited, by fertility problems and a lack of recombination between the wild relative and wheat genomes.

The first barrier to gene transfer from wild relatives is reproductive incompatibility between species, making it difficult to produce interspecific hybrids. The reproductive barrier is often embryo or endosperm abortion after a successful fertilization. It is therefore possible to avoid this abortion using in vitro techniques for example embryo rescue (Gill *et al.* 1981; Valkoun *et al.* 1990).

F_1 interspecific hybrids are often sterile. F_1 are usually sterile because there are no homologous chromosome pairs present, hence the chromosomes cannot successfully pair and recombine. F_1 sterility can be overcome by doubling the ploidy level. Colchicine treatment is often used to double the ploidy of F_1 interspecific hybrids to produce fertile amphidiploids. The wheat/wild relative amphidiploid can then be used as a bridge to facilitate gene transfer from the wild relative into wheat (Saulescu *et al.* 2011). The amphidiploid itself is sometimes useful, for example triticale is a wheat x rye amphidiploid and is now an important crop plant.

The amount of recombination that occurs between the wild relative and wheat genomes within the interspecific hybrid depends on how closely related the wild relative is to wheat.

1.6 Wheats' polyploid nature and an introduction to the Ph1 locus

T. aestivum, hexaploid wheat, (2n = 6x = 42, genomes AABBDD) contains 21 homologous chromosomes pairs, sorted into 7 homoeologous groups, each group containing one homologous pair from each of wheat's A, B, and D genomes (Sears 1954). Therefore, homoeologous group 1, for example contains the pairs 1A, 1B, and 1D, figure 1.6.

Euploid wheat acts cytologically as a diploid and pairing is restricted to true homologues. Hence homoeologous chromosomes do not readily recombine and therefore gene transfer between the different genomes does not happen. To introgress small regions of wild relative chromatin and break linkage drag it is vital to force recombination between the homoeologous wheat and wild relative chromosomes. The chromosomes of wheat and its wild relatives are homoeologous and although they are similar they do not readily pair and recombine (Riley *et al.* 1959). Diploid like meiosis, is controlled by the *Ph* loci, which insures stable chromosome numbers as well as the effective production of gametes therefore safeguarding the fertility of the plant.

The major *Ph* locus *is Ph1*, Pairing Homolog 1, located on chromosome 5B (Okamoto 1957; Sears and Okamoto 1958; Riley and Chapman 1958a; Riley *et al.* 1959). If *Ph1,* is deleted or inactivated, pairing between homoeologous chromosomes can occur (Sears 1976; Griffiths *et al.* 2006; Al-Kaff *et al.* 2008). The deletion of *Ph1* therefore facilitates recombination between the homoeologous chromosomes of the different genomes of wheat. Wheat with a *ph1* deletion and captured wild relative chromosomes can be used to facilitate recombination between the wheat and wild relative chromosomes. *Ph1* can therefore be exploited to produce small wheat wild relative introgressions (King *e*

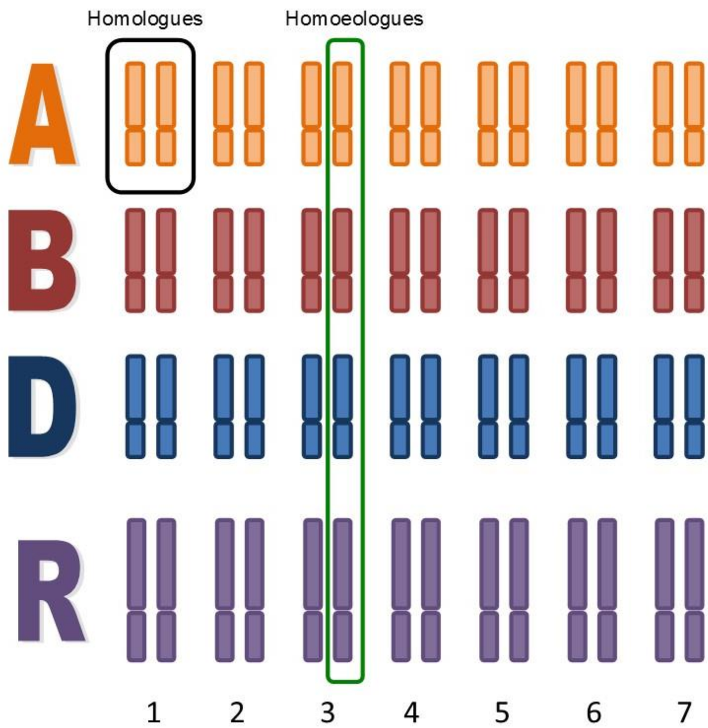


Figure 1.6. A representation of homologous and homoeologous chromosomes. Each bar represents a chromosome. Two bars close together show a pair. Each row is a genome - A, B and D are each of the wheat genomes and R is the rye genome. Each column is a linkage group, containing related chromosomes. The chromosomes within the black box show a homologous pair. The chromosomes within the green box are homoeologues.

t al. 2017). *A* method to produce wild relative introgressions are discussed in detail in chapter 3.

Sears (1977) used deletions to show *Ph1* to be a deletion phenotype. It has taken over 50 years from *Ph1'*s discovery to show that *Ph1* ensures cytological diploidization by promoting homologous pairing, rather than preventing homoeologous pairing and *Ph1* prevents homoeologous recombination by stalling double holiday junctions in paired homoeologous from being resolved as crossovers (Martín *et al.* 2014).

At a molecular level *Ph1's* mode of action is not yet fully understood. *Ph1* is likely to be a defective Cyclin-dependant kinase two-like (*Cdk2*-like) gene or cluster of genes, which alters kinase activity and overall phosphorylation levels (Griffiths *et al.* 2006; Al-Kaff *et al.* 2008; Yousafzai *et al.* 2010). Okadaic acid treatment, which increases phosphorylation levels, replicates the *ph1* mutant phenotype in wheat-rye hybrids (Knight *et al.* 2010). Hence it is probable that the *Ph1* phenotype is caused by a defective *Cdk2*-like gene which reduces phosphorylation by a dominant negative effect (Greer *et al.* 2012; Moore 2015).

Moore (2015) reviews the current work on the *Ph1* locus and shows that *Ph1* has separate effects on recombination and chromosome pairing. Homologous pairing is promoted in three ways; firstly, influencing the conformational changes required in initial pairing, secondly altering telomere bouquet formation, and finally increasing the stringency of independent centromere pairing.

A recent study using a tilling mutants has shown that the TaZIP4-B2 gene that is present within the 5B *Ph1* region is responsible for supressing homoeologous cross overs and therefore recombination in hybrids and the deletion TaZIP4-B2 has been shown to induce the *Ph1* mutant phenotype (Rey *et al.* 2017).

1.7 Fragmentation techniques

The following techniques all induce chromosome breakage and fragmentation. After fragmentation the chromosomes can then randomly join and be repaired creating translocations.

Gametocidal genes are selfish genes that are preferentially transferred from generation to generation. Gametocidal genes persist by causing sterility through chromosome breakage in the gametes that do not contain the gametocidal gene. When the gametocidal induced breakages are not lethal these gametes can be fertilized. The broken chromosomes can then translocate to other chromosomes. Therefore gametocidal genes can be used to induce translocations between wheat and a wild relative containing a gametocidal gene (Endo 1988; Endo 2007). Gametocidal genes are found in a subset of the *Triticeae*, and include species with C, S or M genomes (Endo 2007) ; for example the *Gc2* gene was isolated from the short arm of chromosome 4S in *Aegilops sharonensis* (Friebe *et al.* 2003) and the *Gc3-Cl* gene from chromosome 3C of *Aegilops triuncialis* (Endo 2007). The application of a gametocidal method is limited in a breeding situation. The gametocidal genes are difficult to remove from a population once they are introduced and are often linked to negative traits.

Tissue culture can also be used to induce wheat x wild relative translocations. Wheat is crossed with a wild relative and the resulting hybrid embryo is regenerated using tissue culture. The hybrid embryo is induced to form a callus and grown in tissue culture. The callus is periodically subdivided to grow into distinct adult plants. Chromosomes in the tissue culture callus are unstable resulting in chromosome breakage and translocation. Therefore the adult plants contain different deletions and translocations (Lapitan *et al.* 1984).

Ionizing radiation can be used to induce random breakages in chromosomes and therefore cause translocations (Sears and Okamoto 1958; Sears and Gustafson 1993), e.g. irradiation causes chromosomes in the pollen mother cell to break and they are then randomly repaired. The random translocations are then passed to the gametes. If a plant containing both wild relative and wheat chromosomes is irradiated, gametes containing wheat wild translocations are possible. Sears (1956) managed to successfully use irradiation to introgress a segment of *Aegilops umbellulata* into wheat, the segment carried a gene for rust resistance that hugely benefited the wheat crop in the United States of America until the resistance was overcome.

Irradiation, tissue culture and gametocidal techniques share the same problem. In all cases the chromosome breakages produced are random. The translocation of large regions of chromatin results in problems with linkage drag (see below). The random nature of the chromosome fragmentation can also be problematic as the exchanges are not necessarily reciprocal. Non-reciprocal translocations can have large effects on the viability of a plant as the translocations are unlikely to compensate for genes that are added or lost.

1.8 Wild Relatives success

The most successful use of wild relatives is the 1RS translocation lines. The translocation lines originated from translocations that replaced a chromosome arm of wheat with 1RS of *S. cereale*. The most prevalent 1RS translocation line is 1RS.1BL followed by 1RS.1AL. The 1RS translocation lines were first produced in the early 20th century via natural hybridisation and spontaneous substitution and have been successful because of the presences of genes for disease resistance and increased yield potential (Carver and Rayburn 1994; Singh *et al.* 2009).

Introgressions from wild relatives have been highly successful sources of disease resistance to most wheat diseases (table 1.3). It is possible to produce lines containing multiple introgressions from different wild relatives (Ali *et al.* 2016). Incorporating multiple introgressions from various sources enables pyramiding of resistance genes and the development of stronger and more durable resistance or resistance to multiple diseases.

Wild relatives also contain genes for a wide range of other traits such as abiotic resistance, [e.g. frost hardiness in *T. monococcum* and *Aegilops cylindrica* (Iriki *et al.* 2001; Miller *et al.* 2006), salt tolerance in *Thynopyrum* species], quality traits, [e.g. variation for storage proteins in *T. monococcum* and *Th. ponticum* that may enhance bread making quality], useful agronomic traits, [e.g. a novel dwarfing gene was found in *Th. ponticum* (Chen *et al.* 2012) and there is evidence for potential yield gains from wild relative genes (Wu *et al.* 2006; Song *et al.* 2013; Mohammed *et al.* 2014; Kuzmanović *et al.* 2016)].

Wild relatives have recently been used to combat the emergence of a new strain of stem rust (*Puccina graminis)* called Ug99 (or TTSK). Ug99 is virulent in cultivars carrying most of the current stem rust (*Sr*) resistance genes and therefore was, and to an extent still is, a threat to global wheat production. Wild relatives, however, have been shown to contain novel avriulence genes that confer resistance to Ug99 (Rouse and Jin 2011)

Table. 1.3. A selection of wheat wild relatives and a few examples of the diseases they confer resistance to. Example genes are given.

Species	Disease	Genes	References
Aegilops caudata	Leaf Rust	*Lr57*	(Riar *et al.* 2012)
	Yellow Rust	*LrAC*	(Toor *et al.* 2016)
Aegilops speltoides	Leaf rust	*Lr28*	(Naik *et al.* 1998)
	Powdery mildew	*Pm32*	(Hsam *et al.* 2003)
Amblyopyrum muticum	Powdery mildew	-	(Eser 1998)
Thynopyrum elongatum	Stem rust	-	(Turner *et al.* 2013)
	Fusarium Head blight	-	(Fu *et al.* 2012)
Thynopyrum intermedium	Leaf rust	*Lr38*	(Dyck and Friebe 1993)
	Stem rust	*Sr43*	(Kim *et al.* 1993)
	Stem rust	*Sr44*	(Khan 2000)
	Powdery mildew	*Pm40*	(Luo *et al.* 2009)
	Powdery mildew	*Pm43*	(He *et al.* 2009)
	Wheat streak mosaic virus	*Wsm1*	(Friebe *et al.* 1991b)
	Barley yellow dwarf virus	*Bdv2*	(Gao *et al.* 2009)
Thynopyrum monococcum	Stem rust	*Sr21*	(Gerechter-Amitai *et al.* 1971)
	Stem rust	*Sr22*	(Kerber and Dyck 1973)
	Leaf rust	*Lr63*	(Kolmer *et al.* 2010)
	Powdery mildew	*Pm4*	(Schmolke *et al.* 2012)
Thynopyrum ponticum	Leaf rust	*Lr19*	(Knott 1968; Friebe *et al.* 1994)
	Leaf rust	*Lr29*	(Friebe *et al.* 1996)
	Stem rust	*Sr24*	(Jiang *et al.* 1994a)
	Stem rust	*Sr25*	(Friebe *et al.* 1994)
	Wheat curl mite	*Cmc2*	(Whelan and Lukow 1990)
	Karnal blunt resistance	-	(Vasu *et al.* 2000)
Triticum timopheevii	Stem rust	-	(Allard and Shands 1954)
	Leaf rust	*Lr23*	(Leonova *et al.* 2007)
	Powdery mildew	*Pm27*	(Jarve *et al.* 2000)
	Fusarium head blight	-	(Malihipour *et al.* 2017)

1.9 Linkage drag

Wild relatives are adapted to natural environments and therefore contain many weedy genes alleles, for example the *Br* genes for brittle rachis and *Q* genes that control free threshing that can be detrimental in a crop plant. Wild relative application has been limited because positive (the targeted gene) and detrimental weedy genes are often linked on the same chromosome. This association between positive and negative genes is called linkage drag. When a target gene from a wild relative is introgressed into wheat it 'drags' along a region of wild relative chromatin which may contain deleterious the gene(s).

Linkage drag is a problem in many wild relative introgressions. For example, the 1RS translocation that provides disease resistance also replaces genes important for gluten structure meaning the 1RS translocation cannot be used in cultivars when bread making quality is important (Kumlay *et al.* 2003).

Linkage drag can be overcome by inducing recombination in between the target and deleterious genes. The severity of linkage drag is dependent on the distance between the target gene and the deleterious gene and the size of the introgressed segment. Genes that are a long distance apart are less likely to be linked, and if linked a recombination event to break linkage is probable. Genes close together are more likely to be linked and a recombination event is unlikely. Genes that overlap will be linked and breaking the linkage is impossible. The larger an introgressed segment is, the more likely the segment is to contains deleterious genes. Hence the smaller the introgressed segment the better.

Linkage drag has proved to be a persistent problem in wild relative introgressions because of relatively large introgression sizes and the

difficulty of inducing recombination between the chromosomes of a wild relative and wheat.

Breaking linkage

Linkage can be broken by forcing recombination in between the target gene(s) and deleterious genes. Sears (1981) described a method of reducing the size of introgressed chromatin by crossing two plants with overlapping introgressions. Recombination can occur in the overlap, i.e. between the two wild relative segments, and this will result in the production of lines with smaller introgressions.

1.10 Secale cereale, Rye

To successfully introgress traits to improve wheat a suitable donor species needs to be chosen. While most members of the *Triticeae* have potential to provide beneficial germplasm, this work has chosen to focus on *Secale cereale,* common name rye. Rye is predominantly a diploid member of the *Triticeae* (2n = 14, RR) but some autotetraploid varieties exist (Roseweir and Rees 1962). Rye contains an R genome and as a diploid comprises seven pairs of chromosomes which are homoeologous to the linkage groups of wheat. Unlike wheat, rye is allogamous, meaning rye is self-incompatible and therefore outcrossing is necessary (Lundqvist 1956).

A background of Rye, Secale cereale

Rye is a temperate cereal which was domesticated after wheat, the literature has yet to come to a consensus of the exact time of ryes' domestication, but is known to be between 10,000 and 6,600 years ago (Stutz 1972; Willcox 2005; Fuller 2007). Rye is grown as both a grain and a forage crop. Rye grown for human consumption is chiefly used to produce rye bread, rye whiskey and rye beer. Rye is often a secondary crop to the more valuable grains, such as wheat and barley, and is regularly grown in areas unsuitable for other crops, for example, on marginal land. Rye is

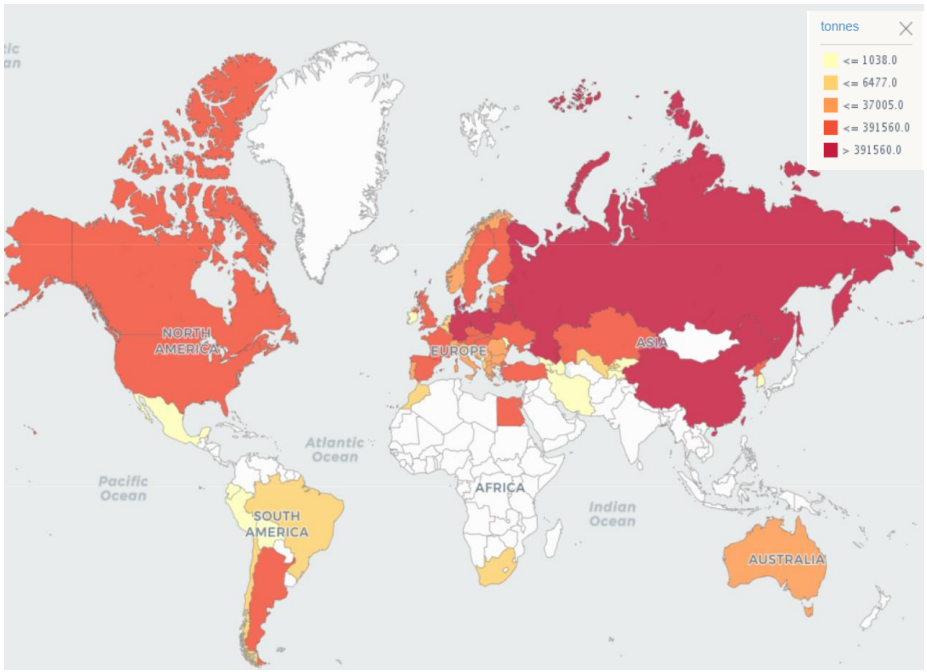
grown primarily in eastern, central, and northern Europe with Germany, Russia, and Poland being the top three producers (FAOstat. http://www.fao.org/faostat). Due to cold and frost tolerance (Fowler and Limin 1987; Erath *et al.* 2017) rye can be grown at high latitudes and altitudes and is an important cover crop (Snapp *et al.* 2005)

In 2016 the global rye grain production was 12.9 million tonnes grown over 4.4 million hectares, whereas wheat produced 749.4 million tonnes of grain on 220.1 million hectares of land. Therefore, rye produced 58 times less grain on 50 times less land than wheat (FAOstat.http://www.fao.org/faostat). Figure 1.7 shows the relative production of rye and wheat in 2016 by country

Rye is arguably the most successful wild relative donor. It is an important crop plant and has therefore been under selection for the demands of modern agriculture. However, rye has retained diversity for the stress tolerance and resistance traits common in the weedier wild relatives. For example, rye is hardy and is tolerant to multiple abiotic and biotic stresses ; Mohammadi *et al.* 2003) including high cold tolerance compared to other winter cereals and is therefore used as a major cover crop across Europe (Limin and Fowler 1987; Webb *et al.* 1994; Madej 1996). Rye also gives relatively high yields in nutrient poor soils (Cakmak *et al.* 1997; Erenoglu *et al.* 1999). Table 1.4 shows a selection of the traits rye is known to posses

Extensive work has been undertaken phenotyping rye as well as introgression lines, addition lines and triticale meaning many R genome traits have been linked to specific chromosomes. The previous work provides an indication of the traits novel introgressions are likely to contain.

Rye



Wheat

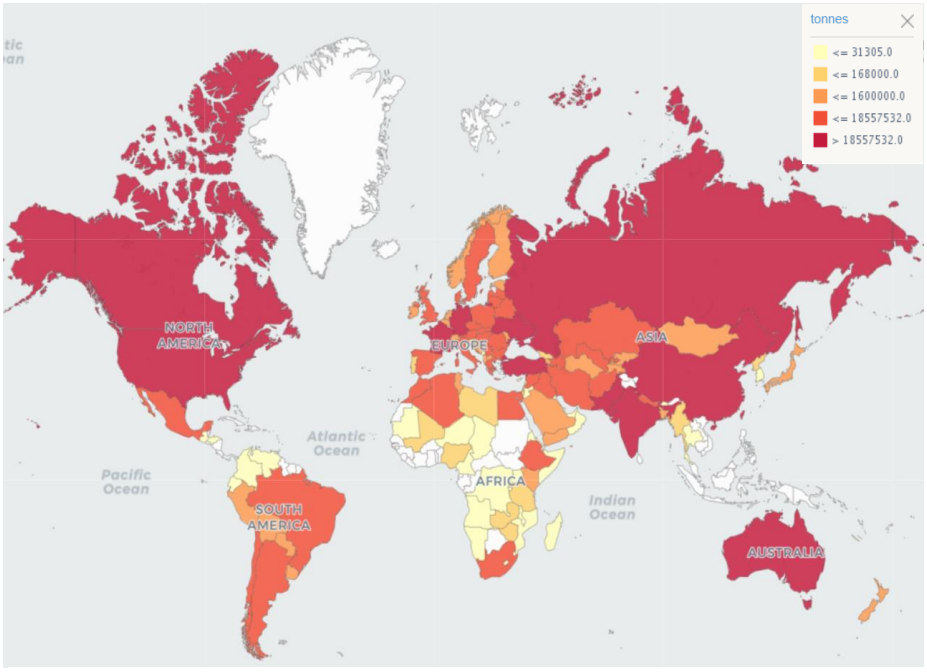


Figure 1.7 Shows the world with each country colour coded based on the amount of cereal grain produced in 2016. The top map shows the amount of rye grain produced and the bottom map shows wheat. Darker colours indicate higher production. To make the maps comparable the colour coding is scaled compared to the total production of each crop because wheat production is ~58 times greater than rye. The figure is adapted from FAOstat see [(http://www.fao.org/faostat/en/#data/QC/visualize) / (http://fenixservices.fao.org/faostat/static/documents/QC/QC_methodology_e.pdf)]

Table 1.4. Shows a selection of traits rye is known to possess. Some key loci have also been noted, along with a relevant reference(s)

Location	Trait	Locus	Reference(s)
1RS	Green Bug resistance	*Gb2*	(Mater *et al.* 2004)
1RS	Leaf Rust Resistance	*Lr26*	(Mago *et al.* 2005)
1RS	Powdery Mildew resistance	*Pm8, Pm17*	(Heun and Friebe 1990; Hanušová *et al.* 1996; Ren *et al.* 2009; Lu *et al.* 2014)
1RS	Drought tolerance	-	(Hoffmann 2008)
1RS	Stripe Rust resistance	*Yr9, YrCn17*	(Mago *et al.* 2005; Ren *et al.* 2009)
1RS	Stem Rust Resistance	*Sr31*	(Mago *et al.* 2005)
1RS	Yield	-	(Singh *et al.* 2009)
1RL	Dwarfing	*Ddw3*	(Stojałowski *et al.* 2015)
2R	Drought resistance	-	(Farshadfar *et al.* 2013)
2R	Increased dietary fibre	-	(Boros *et al.* 2002)
2R	Stem Rust resistance	-	(Adhikari and McIntosh 1998)
2RL	Powdery Mildew resistance	-	(Merker and Forsström 2000)
3RS	Aluminium tolerance	-	(Anioł 2004)
3R	Drought tolerance	-	(Farshadfar *et al.* 2013)
3R	Stem Rust resistance	*Sr27*	(Adhikari and McIntosh 1998)
4R	Aluminium Tolerance	*Alt3*	(Aniol and Gustafson 1984)
4R	Drought resistance	-	(Farshadfar *et al.* 2013)
4R	Frost tolerance	-	(Erath *et al.* 2017)
4R	Fusarium Head Blight resistance	-	(Kalih *et al.* 2015)
4R	Powdery mildew	-	(An *et al.* 2013)

5RL	Soil Borne cereal mosaic virus Resistance		(Erath *et al.* 2016)
5R	Aluminium tolerance	-	(Manyowa and Miller 1991)
5R	Drought tolerance	-	(Farshadfar *et al.* 2013)
5R	Dwarfing	*Ddw1*	(Korzun *et al.* 1996)
5R	Increased dietary fibre	-	(Boros *et al.* 2002)
5R	Frost tolerance	*Fr-R2*	(Erath *et al.* 2017)
5R	Fusarium Head Blight resistance	-	(Kalih *et al.* 2015)
6R	Drought resistance	-	(Farshadfar *et al.* 2013)
6R	Increased dietary fibre	-	(Boros *et al.* 2002)
6RL	Hessian Fly resistance	-	(Friebe *et al.* 1991a)
6RL	Nematode resistance	*CreR*	(Dundas *et al.* 2001)
6RL	Powdery Mildew resistance	*Pm20*	(An *et al.* 2015)
6RS	Aluminium Tolerance	*Alt1*	(Aniol and Gustafson 1984)
7R	Aluminium Tolerance	*Alt4*	(Niedziela *et al.* 2014)
7R	Drought tolerance	-	(Farshadfar *et al.* 2013)
7R	Dwarfing	*Ddw2*	(Melz 1989)
7R	Frost tolerance	-	(Erath *et al.* 2017)
7R	Fusarium Head Blight resistance	-	(Kalih *et al.* 2015)
7R	Wheat Spindle Streak Mosaic Virus	-	(Erath *et al.* 2016)

Rye germplasm within wheat

The capture of rye germplasm into wheat was started in the late 19th century, with the first documented wheat rye interspecific hybrids (Wilson 1873; Carman 1886; Roberts 1904). The next advance was the report of spontaneous 5R(5A) substitution lines (Kattermann 1937; O'Mara 1947; Riley and Chapman 1958b).

Rye germplasm has been transferred into wheat using multiple methods. These include direct crossing between wheat and rye, using wheat rye substitution lines, using triticale as a gene bridge to back cross to wheat and via the fragmentation techniques described in section 1.8 [gametocidal genes (Masoudi-Nejad *et al.* 2002), tissue culture (Lapitan *et al.* 1984) and ionizing radiation (Sebesta and Wood 1978)] . The techniques mentioned above share a common twofold problem; they introgress large segments of rye chromatin and they do not induce recombination, resulting in problems with linkage drag and the loss of wheat genes.

Adoption of rye germplasm has become wide spread because of the development of 1BL/1RS translocation lines and to a less extent 1AL/1RS translocations and 1B(R) substitutions. The short arm of chromosome 1 from rye is the most prolific and successful use of a 'wild' relative to improve wheat breeding lines. There are four major sources of 1R translocations and substitutions; two developed in Germany in the 1920-30s, one developed in japan in the 1960s and one developed in the USA in the 1970s. One of the German translocations was produced from the rye cultivar Petkus by repeated backcrossing, whereas the other three 1R sources were produced from backcrossing to triticale (Rabinovich 1998). The German variety produced by Georg Riebesel, from Petkus rye was the first, and is currently the most widely developed 1RS source (Crespo-Herrera *et al.* 2017). The 1Bl.1RS and 1AL.1RS translocations are prevalent across the globe in wheat breeding programs (Lukaszewski 1990; Schlegel and Korzun

1997; Rabinovich 1998; Zhou *et al.* 2003). According to Schlegel (2018) there are over 1,000 varieties with the 1RS.1BL translocation, ~100 varieties with the 1RS.1AL translocation, and about 30 varieties carrying the 1R(1B) substitution. In total there are 2,470 wheat cultivars or experimental lines containing wild relative introgressions and hence 1RS cultivars account for just under half.

The 1RS translocation from Petkus has been a successful source of disease resistance genes. Originally, Petkus 1Rs was used for the following resistance genes *Sr31*, *Lr26*, *Yr9* and *Pm8*. The genes above are no longer effective (Bartos 1996; Pretorius *et al.* 2000) but the translocation is still present in elite lines, likely due to a yield advantage. (Villareal *et al.* 1998; Singh *et al.* 2009)

There is evidence for varying alleles on different rye cultivars; therefore there has been considerable work to produce novel sources of 1RS translocations. (Ren *et al.* 2009; Tang *et al.* 2009; Lu *et al.* 2014; Qi *et al.* 2016). Although 1RS is still likely to continue to be a focus of most work in the field, rye contains another six and a half chromosomes that contain potentially useful genes (see table 1.4). Hence to gain the maximum benefit, a rye crossing program should not actively select a single chromosome.

Moreover though rye germplasm have been extensively used the majority of work has focused on 1R therefore only a fraction of rye's potential has been exploited, however as rye contains another 6 and a half chromosomes and there is still variation in 1RS that can be captured (Qi *et al.* 2016). The source and position of the introgression in the wheat genetic background can alter the resulting phenotype (Kumlay *et al.* 2003; Kim *et al.* 2004). Therefore, work introgressing novel regions of rye as well as further work introgressing known beneficial regions is justified.

The rye genome

The rye genome is the largest of all the diploid Triticeae (~7.9 Gbp; Bartoš et al. 2008), with the majority (90%) of the genome consisting of repetitive sequences (Flavell et al. 1974). Rye is an outcrossing (allogamous) species and therefore shows a high level of nucleotide diversity (Bauer et al. 2017). Rye has undergone a series of genome reorganisations that has disrupted the collinearity between wheat and rye in all but homoeologous group 1. The rearrangements in rye were originally revealed by restriction fragment length polymorphism (RFLP) mapping by Devos et al. (1993a) then expanded upon with a high density SNP array by Martis et al. (2013) and have recently been confirmed by the production of a whole genome sequence of rye by Bauer et al. (2017) Martis *et al.* (2013) proposed that a series of five rearrangements have occurred in rye since the original Triticeae progenitor. The first rearrangement occurred before the divergence from the wheat A genome progenitor and is therefore present in the A genome of hexaploid wheat, whereas the B and D genomes of wheat have not undergone rearrangements, see figure 1.8 from Martis *et al*. (2013)

During the course of this PhD a whole-genome draft sequence of rye has been produced by Bauer et al. (2017), which will be a valuable resource in future projects> In particular if the genome assembly was available at the start of this project it would have expedited marker design.

Figure 1.8 The rye genome rearrangements compared to the Triticeae progenitor from Martis *et al.* (2013).
"Rye genome reorganizations occurring in the common ancestor of rye and wheat (translocation between chromosomes 4 and 5) and divergence of the two lineages are postulated. Three of the five translocations that occurred after the split of wheat can be ordered, while for two the order cannot be deduced. They may have occurred in parallel or consecutively.
Genes" Martis *et al.* (2013

1.11 Techniques to identify introgressions

A successful modern breeding scheme needs an accurate and high throughput method of identifying and characterising introgressions. A successful method needs to be able to accurately show the presence of an introgression, its characterisation, and be able to deal with the large number of plants produced. As well as being able to track the introgression through generations.

There are several different methods of identifying introgressions that have used to with cereal crops. These methods can be split into two major categories; firstly, methods using cytogenetic approaches that visualise the crops genome and secondly more modern methods using molecular markers to genotype the crop.

Cytogenetic techniques

Much of the early work investigating the wheat genome was undertaken using a technique known as C-banding (Gill and Kimber 1974a). C-banding uses a Giemsa stain that binds to regions of heterochromatin producing a heterochromatin banding pattern. The heterochromatin banding pattern differs depending on linkage group and genome and therefore individual chromosomes can be identified, e.g. it is possible to identify wheat to rye translocations using C-banding (Lukaszewski and Gustafson 1983). C-banding has a limited resolution and therefore cannot identify small changes to a chromosome. The C-banding technique itself is also challenging because of the need to produce high quality metaphase spreads and the time and expertise needed to analyse the resulting images.

Fluorescence in situ hybridization (FISH), developed in the early 1980s (Langer-Safer *et al.* 1982), is now widely used in cereal research. FISH is an intermediate between the classical cytogenic techniques such as C-banding and molecular techniques. FISH takes a section of DNA and attaches a

fluorescent probe which then identifies specific targets. FISH can be used to karyotype a genome, whereby a fluorescently labelled probe is hybridised to complementary regions in a metaphase chromosome spread, producing banding patterns on the different chromosomes. The banding pattern changes dependant on the sequence of DNA used as a probe and the target chromosomes. The banding patterns can then be used to identify the different chromosomes as well as showing the presence of an introgressed region of chromatin. FISH has similar drawbacks to C-banding because of the need for high quality metaphase spreads and the difficulty of analysing the images produced. FISH has some key advantages over C-banding, that include a higher resolution, sensitivity, and speed (Devi *et al.* 2005). FISH can indicate the presence of a specific section of DNA of interest. FISH can also use different fluorochromes to identify multiple targets simultaneously.

Genomic *in situ* hybridization (GISH), is a modified form of FISH and particularly useful in polyploid species or introgression lines. GISH takes the complete genome of an organism and uses it as a fluorescently labelled probe (Schwarzacher 2003). In an allopolyploid species GISH can be used to indicate relationships to progenitor species and can be used to discriminate between genomes. GISH is exceptionally useful for identifying introgressions as the donor species can be used as a probe (Devi *et al.* 2005).

Molecular Genotyping

The molecular genotyping of wheat is a considerable challenge because of wheat's exceptionally large and repetitive genome and its polyploid nature. Hexaploid bread wheat has a huge haploid genome of ~15,000 megabase pairs (Zimin *et al.* 2017) which is around five times the size of the haploid human genome, ~ 3,300 megabase pairs (Venter *et al.* 2001; Jain *et al.* 2018). Bread wheat is a hexaploid meaning each set of chromosomes has three copies, one from each progenitor genome. Each chromosome set is similar but contains important differences. The similarities cause difficulty in

assigning markers or sequence reads to the correct chromosomes and makes designing genome specific markers difficult.

The first molecular markers were isozymes, syn. allozymes. lsozymes are a protein based marker system, developed in the 1960s (Hubby and Lewontin 1966), which recognises protein variants in enzymes using gel electrophoresis. In the 1970s to the 1990s isozymes were used extensively to genotype cereals including mapping both wheat and rye (Hart 1983; Jaaska 1983; Vaquero *et al.* 1990). DNA based techniques have succeeded isozymes as they more directly detect genetic variation.

The 1980s saw the introduction of restriction fragment length polymorphisms (RFLPs) (Botstein *et al.* 1980). RFLPs exploit restriction endonucleases that cleave DNA at specific restriction sites. Changes in the restriction site results in different fragment lengths and therefore fragment banding patterns are used to indicate differences between genomes. RLFPs were the first method to successfully associate a trait with markers (Kerem *et al.* 1989). RFLP studies have been successfully used to map wheat (Chao *et al.* 1989; Devos *et al.* 1992; Devos *et al.* 1993b) and have been associated with many wheat traits. However, RFLP maps lack the resolution of more modern techniques. Devos *et al.* (1993a) used RLFPs to compare the orthologous gene order of rye and wheat genomes showing many large chromosomal rearrangements.

A key advantage of all the molecular techniques mentioned above is they require limited prior knowledge of the genome sequence to be developed and employed. The following techniques, however, require extensive prior sequence knowledge of the genome being investigated.

Ishikawa (2007) developed a marker method based on EST-PCR (Expressed sequence tag -PCR) adapted for use on hexaploid wheat. EST-PCR uses the known location of an expressed sequence to anchor a PCR marker. The

multiple gene copies found in hexaploid wheat mean multiple products are amplified simultaneously making it difficult to assign a marker to the correct genome. To overcome the problem, Ishikawa (2007) developed PCR-based Landmark Unique Gene (PLUG) markers which incorporate intron regions in the PCR product that are more polymorphic and thus allowing differentiation between the products from each genome. PLUG markers have been used to further resolve the comparative orthologous gene order of the wheat and rye genomes (Li *et al.* 2013).

Most of genotyping work being undertaken currently uses single nucleotide polymorphisms (SNPs). SNPs are single nucleotide base pair variants at a specific place in the genome. The sequence surrounding a SNP is used to design markers that identify the SNP alleles. SNPs are suited for use in automated and high throughput systems with companies, such as Illumina (www.illumina.com) and Axiom (www.thermofisher.com), producing SNP arrays for use in commercial breeding. SNPs have been produced that cover the whole wheat genome and have been used to study changes in wheat diversity (Wang *et al.* 2014).

Sequencing

Though technically not a molecular marker, sequencing is arguably the most informative method of genotyping and assessing variation as it provides the highest possible resolution with reduced bias. The use of sequencing to test for variation has been limited by technical difficulties and cost. The move away from Sanger sequencing with the development of next-generation sequencing technologies has led to higher-throughput, faster and cheaper sequencing. As next-generation sequencing improves it is likely sequencing will play a larger role in genomic studies and trait identification.

A near complete sequence of the hexaploid wheat genome assembly, Triticum 3.0, has recently been completed (Zimin *et al.* 2017) by a small group of US scientists who completed the genome before the International

Wheat Genome Sequencing Consortium (IWGSC), a giant consortium of 2,100 members that started work on the genome in 2005. IWGSC have recently released a fully annotated reference genome for wheat (IWGSC 2018). Further work to produce a wheat pangenome that incorporates the genomes found in other accessions is being undertaken (Montenegro *et al.* 2017; Uauy 2017). The availability of a complete wheat genome will expedite marker design and make studying wheat genomics easier.

Genotyping Choices

In the work described in this thesis is a combination of both cytogenetic and molecular approaches has been employed. GISH was chosen due to its ability to unambiguously discern the presence of large regions of wild relative chromatin and therefore will be used as a control for the higher throughput molecular work.

Two high throughput SNP based methods were chosen for molecular genotyping. The first SNP system chosen was the Axiom® genotyping array, developed specifically for 10 wild relatives, which are *Am. muticum, Ae. speltoides, Ae. caudata*, *T. timopheevii*, *T. urartu*, *S. cereale*, *Th. bessarabicum*, *Th. elongatum, Th. intermedium and Th. ponticum*. The Axiom® wild relative array contains 35K SNPs with polymorphisms for the 10 wild relatives. All 35K SNPs that form the wild relative array were taken from the Axiom® 820K SNP array (Winfield *et al.* 2016).[The data set for the Axiom® 820K array is available from www.cerealsdb.uk.net (Wilkinson *et al.* 2012)] The Axiom® wild relative array allows for high throughput identification of regions of wild relative chromatin.

The second SNP system chosen was Kompetitive Allele Specific PCR (KASP), The KASP system was chosen because of the techniques flexibility and its ability to design novel markers that will allow the production of genome specific primers that can identify both wild relative introgressions and the different genomes of wheat (LGC, www.lgcgroup.com/).

Wheat Improvement Strategic Programme (WISP)

The work undertaken in this thesis was originally part of a larger project, The Wheat Improvement Strategic Programme, WISP, funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and is now part of the follow on programme Designing Future Wheat (DFW). WISP was a collaborative program with members from the following institutions: John Innes Centre, National Institute for Agricultural Botany, University of Nottingham, University of Bristol, and Rothamsted Research. DFW is a wider ranging programme involving more researchers from those institutes listed above but also now including the Earlham Institute and the Quadram Institute.

The goals of the WISP project were and the DFW are:

“

- Understand the genetics behind factors limiting grain yield, such as drought tolerance, plant shape and resistance to pests and diseases.
- Identify new and useful genetic variation from related species and sources of wheat germplasm not adapted to target environments.
- Cross wheat lines to produce germplasm that allows the identification of genes influencing key traits.
- Generate a database of genetic markers, for use in precision breeding

“ Goals quoted from http://www.wheatisp.org/Consortium/WISP.php.

The WISP project was subdivided into 4 pillars, (Landraces, Synthetics, Wild relative introgression and Elite Wheats) and themes (Phenotyping and Genotyping). The first three pillars, Landraces, Synthetics and Wild relative introgression aimed to improve wheat by exploiting different sources of diversity. The aim of the two themes was to improve techniques and exploit and utilise the pillars by developing methods to effectively genotype and

phenotype the material produced. The fourth pillar was undertaken by private breeders combining the work of the other pillars and themes into elite cultivars of wheat.

This thesis is part of the alien introgression pillar in close collaboration with genotyping and the plant material produced will be available for phenotyping.

1.12 Aims and Objectives

The goal of this field of research is to produce improved cultivars of elite wheat. To facilitate this goal this project intends to expand the diversity present in hexaploid bread wheat, by introgressing rye germplasm into a bread wheat background. The rye accessions from the United States Department of Agriculture (USDA) were chosen because they showed high levels of tolerance in a recent (unpublished) rust trail. The rye accessions provided by KWS were chosen on the recommendation of the KWS, Global Lead Scientific Affairs,Victor Korzun, because of the lines show potential to be useful for breeding purposes.

To be of maximum use in future breeding projects the introgressions produced need to be small, which reduces linkage drag, this will be achieved by using *ph1* mutants to create the initial introgressions and then further crossing as in Sears (1981) to reduce the introgressions size (as described above) .

This thesis employs a 'shotgun' approach to producing introgressions to include the complete rye genome. Using a 'shotgun' approach means the introgressions produced are random, not targeted. The shotgun approach was chosen to limit bias and aims to capture novel rye introgression that may have been selected against or not identified in previous work. Due to the random nature of the shotgun approach and the large number of lines

produced, it is necessary to develop high throughput methods that span the whole wheat and rye genomes, to genotype the material produced.

The genotyping aims to use a combination of GISH and SNP based platforms to identify introgressions reliably and quickly and to accurately track introgression through the breeding program. With the aim to characterise the introgression produced identifying both the size and position of introgressions. Moreover, the genotyping approach needs to be flexible and robust enough to track introgressions through lines with changeable chromosome numbers and unpredictable chromosome fragmentation. The secondary aim of the genotyping work was to produce a map of the rye genome that will be used in to assign markers in future work and can be used to compare the relative genome structure of wheat and rye

Once lines with single introgressions have been produced they will be self-fertilised until homozygous lines are produced. The homozygous introgression lines will then be freely available with the intention to be of use in breeding programs and future phenotyping studies, therefore providing a source of novel germplasm and traits.

2 General Materials and Methods

Genomic DNA Extraction

3-5 pieces of young leaf (2-4cm) was harvested, placed in 2ml round bottom microcentrifuge tubes and freeze dried for 17 hours (Christ alpha 1-4 LD plus). Freeze dried samples were ground to dust with a tissue lyser (Qiagen TissueLyser II). DNA Extraction buffer (appendix 1) was preheated to 65°C. 800 µl was added to each sample and the samples incubated at 65°C for 1 hour. Samples were then cooled to room temperature, spun at 13,000 rpm for 5 minutes and the supernatant transferred to a new 2 ml microcentrifuge tube. 400 µl of 6M ammonium acetate (cooled to 4°C) was added to the sample, mixed, then kept on ice for 30 minutes before an equal volume of 1:1 phenol/chloroform was added and mixed. The samples were then spun at 13,000 rpm for 5 minutes and the supernatant again transferred to a new 2 ml microcentrifuge tube. DNA was precipitated with 600 µl iso-propanol at -20°C overnight. DNA was pelleted by centrifuging at 13,000 rpm for 15 minutes and the supernatant removed. The DNA pellet was washed 3 times in 70% ethanol. The ethanol was completely removed, and the DNA suspended in 1XTE. The extracted genomic DNA was stored at -20°C for later use, the same DNA extraction protocol was used for molecular and cytogenetic work with the only difference being for molecular work the phenol-chloroform cleaning step was removed.

Complete list of Plant Materials

Plant material

The euploid hexaploid wheat varieties obtained from the Germplasm Resource Unit at the John Innes Centre were, Paragon (WPGS id#23201), Chinese spring (WPGS id#28124), Pavon 76 (idPlant: 20448, GRU Store Code: W7193) and Highbury (idPlant: 15111, GRU Store Code: W1576)
The *ph1* mutant wheat accessions obtained from the Germplasm Resource Unit at the John Innes Centre were Chinese spring (P208/535 and 84) and Paragon (P208/514, 2, 11 and 112). Eight accessions of rye were obtained

from the USDA genebank {390382, 426170, 428373 (Petkus), 578092} and three rye accessions were obtained from KWS (107, Brasetto and Palazzo)

A complete set of Imperial rye addition/substitution lines in a Chinese Spring background were obtained from Germplasm Resource Unit at the John Innes Centre {plant codes:1R[WPGS (id#28191)], 1RL[WPGS (id#28192)], 1RS [WPGS (id#28193)], 2R[WPGS (id#28198)], 2RL [WPGS (id#28199)], 3R[WPGS (id#28201)]. 3RS[WPGS (id#28202)], 4R[WPGS (id#28203)], 4RS[WPGS (id#28205)], 4RL[WPGS (id#28226)], 5R[WPGS (id#28206)], 5RS[WPGS (id#28208)], 5RL[WPGS (id#28228)],6R[WPGS (id#28209)], 6RS[WPGS (id#28213)], 6RL[WPGS (id#28212)],7R[WPGS (id#28214)], 7RS[WPGS (id#28216)] 7RL[WPGS (id#28215)] }.

3 Germplasm Development

3.1 Germplasm introduction

Ryes quality problem

The usefulness of rye introgressions have been limited through an associated decrease in grain quality and grain processing quality due to linkage drag. The 1RS translocation typically results in a sticky and weak dough, due to the introduction of the *Sec-1* locus , that encodes for a gluten homologue and the subsequent loss of wheat genes. Wheat storage proteins, glutenins and gliadins, are therefore replaced with rye storage proteins, secalins, decreasing grain quality (Graybosch 2001).

The linkage between disease resistance and poor grain quality has been shown to be surmountable by inducing recombination between the rye translocation and wheat (Sears 1977; Anugrahwati *et al.* 2008). Recombination is induced between homoeologous chromosomes by the exploitation of the *Ph* loci.

Crossability of Wheat and Rye

The success of wheat rye hybridisation is affected by the varieties of wheat used. Rye hybridisation to wheat varieties with poor crossability set seed in less than 5% of crossed florets, whereas in highly crossable wheat varieties crossed florets set seed more than 50% of the time (Riley and Chapman 1967). Crossability is known to be controlled by two recessive loci *kr1* and *kr2* [reviewed by Molnár-Láng (2015)].

Reasoning for wheat variety selection

The hexaploid spring wheat variety Paragon was chosen as a background for the majority of introgression lines. Paragon was selected because it had been an elite UK variety and is therefore suited for trait analysis. Paragon is crossable to multiple wild species (Moore 2014), and has an available *ph1* deletion line, moreover it is a spring wheat facilitating more crossing

seasons per year than a winter wheat. The wheat varieties Chinese Spring, Highbury and Pavon were also used as the wheat background, to a lesser extent, because they are readily crossable, and *ph1* deletion lines are available. However, they lack the elite pedigree of paragon. Using multiple wheat backgrounds was deemed necessary in order to increase the chances of successfully producing wheat-rye hybrids. This was because the rye accessions chosen had not been previously used to produce wheat-rye hybrids and because the accession if rye and the variety of wheat is known to alter compatibility using multiple varieties of wheat increased the chances of success.

The rationale for the selection of the rye accessions if described in section 1.12 (page 40)

Crossing aims

The *Ph1* Locus has been shown to control homoeologous pairing and recombination, discussed in detail in section 1.6 (pages 16 and 17). King *et al.* (2017) demonstrated the effectiveness of a wild relative breeding program exploiting *Ph1*, by creating interspecific hybrids of hexaploid bread wheat and *Am. muticum*. It was shown possible to introgress segments of *Am. muticum* into wheat. Introgression was possible without a *ph1* mutant because *Am. muticum* carries a gene(s) which suppress the *Ph1* locus (Dover and Riley 1972). Here we describe a program adapted from King *et al.* (2017) using a *ph1* mutant wheat enabling introgression from rye, a wild relative that does not suppress the *Ph1* locus.

3.2 Germplasm Materials and Method

Plant growth and treatment

Plant material

The euploid hexaploid wheat varieties, Paragon (WPGS id#23201) and Chinese spring (WPGS id#28124) were used as well as their *ph1* mutant

accessions (Chinese spring P208/535 and 84: Paragon P208/514, 2, 11 and 112) all wheat accessions were obtained from obtained from the Germplasm Resource Unit at the John Innes Centre. Eight accessions of *Secale cereale* were used: accessions 390382, 426170, 428373 (Petkus), 578092 and Blanco were obtained from the USDA genebank; acessions 107, Brasetto and Palazzo were obtained from KWS.

Seed storage

All Seed was stored at 3°C, at low humidity and in the dark [seed store supplied by Pitkin and Ruddock limited (http://www.pitkin-ruddock.co.uk)].

Germination

Before germination seed was stored, as described above, for a minimum of four weeks to break dormancy.

Seed germination for in situ techniques

Seed to be used in *in situ* techniques was germinated in petri dishes, onto damp filter paper, in a laboratory under natural light. After root tips were collected (section 4.2), seed was transferred to soil (John Innes F2S) in 5 cm trays. Plants in trays were grown in glasshouse conditions (Appendix 1) for 7 days until vernalisation, see below.

Germination of seed not used in in situ techniques

Seed not used in *in situ* techniques was germinated in soil (John Innes F2S) in 5 cm trays. Plants in trays were grown in glasshouse conditions (Appendix 1) for 7-10 days until vernalisation, see below.

Shrivelled Seed Embryo Rescue

Shrivelled seed was softened and sterilised in 5% sodium hypochlorite solution, ClNaO (appendix 1). Each seed was treated with 1ml of 5% ClNaO for 15 minutes, while being shaken. Seeds were rinsed with sterile deionised water three times and shaken between rinses. The seed was then left in

sterile deionised water for a minimum of 6 hours and a maximum of 2 days to soften before embryo dissection. After sterilisation the embryo was removed from the shrivelled seed under sterile conditions and transferred to sucrose media (appendix 1). Sucrose media was produced in 10L batches. 80g of agar was dissolved in 4.5L of purified water. 44.1g of Murashige and Skoog dried medium, MS0, and 300g of sucrose were dissolved in 5L of purified water and the pH was adjusted to pH5.8 using 10M sodium hydroxide and 1.0M hydrochloric acid. The Murashige and Skoog and sucrose solution was mixed thoroughly with melted agar while still hot. 50ml of the sucrose media was decanted into 60mm x 60mm x 80mm, heat proof sealable plastic containers and immediately autoclaved.

The embryo on sucrose media was left in a growth room with 24 hour light at 20°C until large enough to transfer to soil (Figure 3.1). Rescued embryos were transferred to soil (John Innes F2S) in 5 cm trays and then grown under glasshouse conditions (Appendix 1) until transferred to vernalisation, see below.

For all types of germination once plants were transferred to soil, they were watered daily by hand with HortiMix Standard (Hortifeeds) nutrient solution (Appendix 1).

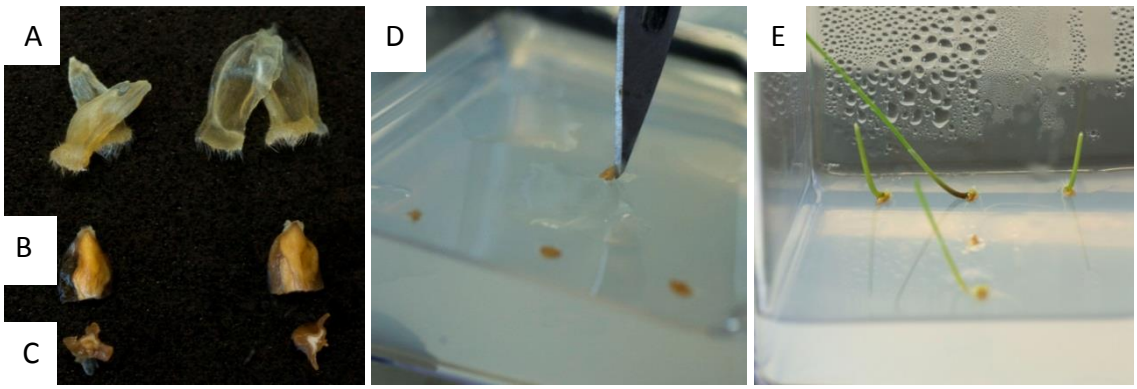


Figure 3.1 The stages of embryo rescue. A, B and C show a dissected seed, A is the bran, B is endosperm and C is the embryo. D shows embryos being placed in sucrose solution. E shows germinated embryos ready to be transferred to soil

Vernalisation

After germination, plants were kept in a growth room at 6-8°C, with a 16-hour light period. The wheat stocks used were spring wheats and given four weeks vernalisation, whereas the rye needed eight weeks to fully vernalise. To account for the varying proportion of rye chromatin present in the introgression lines vernalisation time was varied depending on the lines progression though the crossing scheme. Unless genotyping data was available F_1 and BC_1 were vernalized for eight weeks, BC_2 and BC_3 for six weeks and BC_4 and above for four weeks. When genotyping data was available genotypes with more than six rye chromosomes were vernalised for eight weeks, genotypes with between three and five rye chromosomes were vernalised for six weeks and genotypes with less than two rye chromosomes were vernalised for four weeks.This approach for vernilation was taken to allow larger groups of plants be bulked together and because the presence of vernalisation genes was not known in the backcross generations During vernalisation plants were watered daily by hand with HortiMix Standard (Hortifeeds) nutrient solution (appendix 1).

Plant growth

After vernalisation plants were transferred to individual 2l pots in John Innes No 2 soil and drip fed with HortiMix Standard (Hortifeeds) nutrient solution (appendix 1) and grown under glasshouse conditions (appendix 1).

3.2.1 Crossing scheme

The crossing scheme is shown in figure 3.2, and is adapted from Grewal *et al.* (2017). The crossing scheme used an initial cross between *S. cereale* and a *ph1* mutant wheat to produce a F_1 wheat-rye interspecific hybrid lacking

Ph1, henceforth referred to as F_1. The F_1 was then crossed to a euploid wheat to produce a backcross 1 (BC_1). The BC_1 was then repeatedly backcrossed and genotyped using methods described in chapters 4 and 5, to produce $BC_{(N)}$ plants that contained a single heterozygous introgression (N is the number of backcrosses necessary to do this). The $BC_{(N)}$ was then self-fertilized and genotyped (using methods described in chapters 4 and 5), until a single homozygous introgression was obtained, producing a $BC_{(N)}F_{(n)}$ (n is the number of rounds of self-fertilisation necessary to produce a homozygous introgression.

Complications during the crossing scheme

The crossing scheme depicts and ideal situation wherein during the production of the BC_1 the chromosome number is restored to the 42 present within hexaploid wheat. The chance of returning to 42 wheat chromosomes (with some containing rye introgressions) in the initial backcrosses is low, because the genome composition determined by the random assortment of chromosomes at meiosis and requires the rye chromosomes to be randomly lost. Therefore, until characterisation the actual chromosome number and genome composition of the backcross lines is unknown. Secondly the crossing scheme places stress of the wheat and rye genomes that can cause chromosome fragmentation and the production of chromosomal aberrations.

The crossing scheme described above assumes the rye accession is diploid and therefore has a single chromosome from each homologous group in the F_1. During the course of this project cytogenetic analysis showed that the rye accession390382 was tetraploid and therefore contained two chromosomes from each homologous group in the R genome in the F_1.

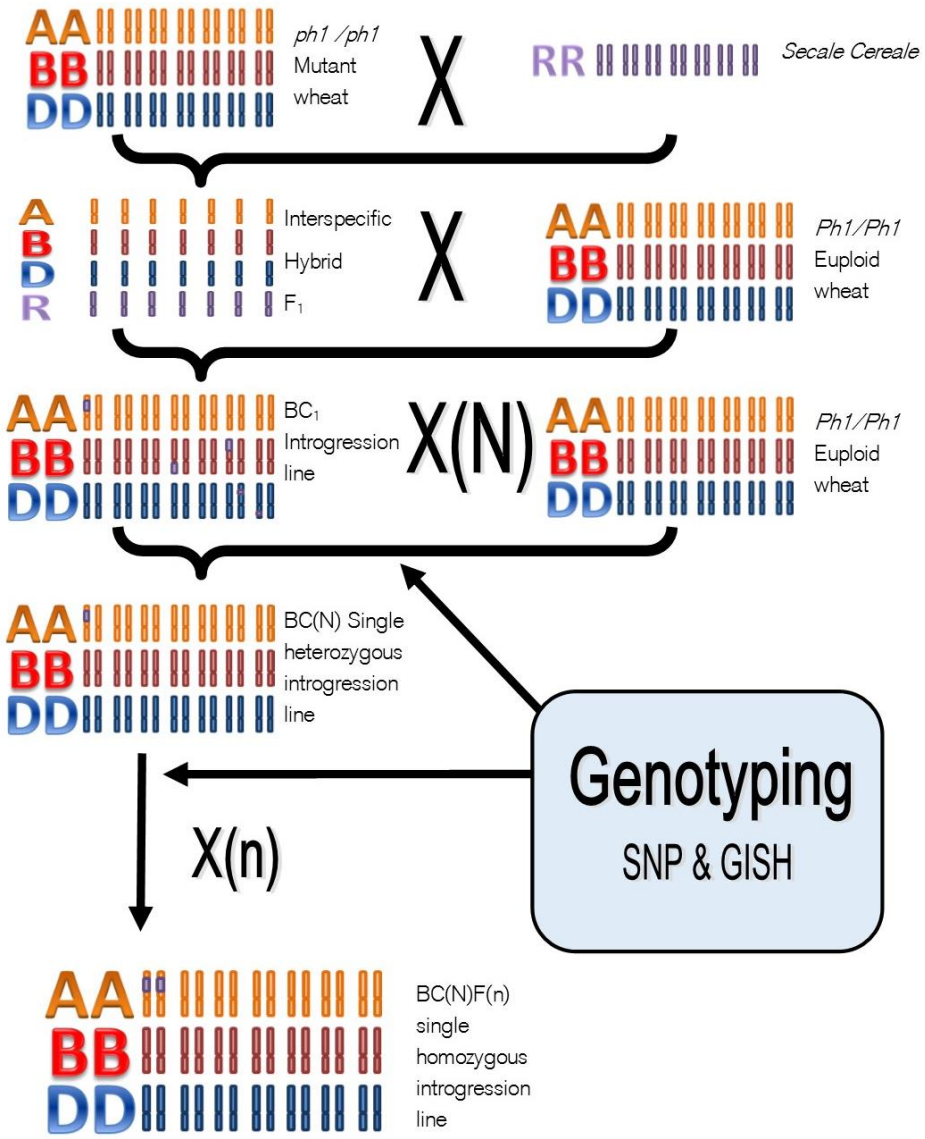


Figure 3.2. A Simplified Crossing scheme, A single bar shows each chromosome. Two bars close together show a homologous pair. Each genome is coloured the AA, BB and DD genomes of wheat being orange, red and blue respectively. The R genome of rye is coloured purple. The initial cross is between *ph1* mutant wheat rye producing a F_1 interspecific hybrid of wheat and rye. The F_1 contains a single chromosome from each linkage group from each genome of the A, B, D and R genomes. The F_1 does not contain homologues only homoeologs and therefore homoeologs are forced to pair at meiosis. This is facilitated by the lack of *ph1.* F_1 plants are backcrossed to euploid wheat, containing functional *Ph1,* to produce a BC_1. BC_1 contain multiple regions of rye and therefore undergoes a process of backcrossing and selection until a BC(N) is produced with a single heterozygous introgression. N is the number of rounds of backcrossing needed obtain a single introgression. BC(N)s are then self-fertilised and selected until a homozygous introgression is produced. Selection for both steps is done using SNP based genotyping (chapter 4) and/or cytogenetic techniques (see chapter 5).

Emasculation

Heads were emasculated 1-3 days before anthesis, while the anthers were still green and before pollen release, preventing self-fertilization. The central three florets were removed from each spikelet leaving the primary and secondary florets. The anthers were then removed from the primary and secondary florets, taking care not to damage the stigmas. The head was then covered in a glassine bag to prevent uncontrolled pollination.

Pollination

Recently matured anthers were selected from the chosen male parent. Anthers were gently removed from the male parent using fine-nose tweezers. The anther was shaken above a receptive stigma on the female parent (stigmas were judged as receptive when they appeared fluffy. After pollination the head was covered in a glassine bag until harvest.

Drying out and Harvest

When plants started senescing their nutrient solution supply was turned off and the plants left to dry. Once the drying finished and the plants had become brittle and yellow the heads were removed using scissors.

Threshing

Self-fertilised seed was machine threshed using a HALDRUP LT-20(from HALDRUP www.haldrup.net). Heads were harvested as above, then threshed according to the Haldrup standard practice. Crossed seed was threshed by hand because it was too fragile to mechanically thresh.

The initial cross and production of interspecific hybrids

F_1s were produced using *S. cereale* as the male parent and *ph1* mutant wheat as the female parent. The *ph1* mutant was emasculated as described above. Once the *ph1* mutant stigmas mature and became receptive, i.e once the stigma became fluffy shown in figure 3.3, they were pollinated with *S. cereale* as described above.

Paragon

Chinese Spring

Figure 3.3. Images of receptive stigma in the two wheat cultivars used in the crossing scheme. The lemma has been removed from the spikelet to reveal the stigma.

Crossing F_1 hybrids

F_1 hybrids were grown to maturity and used as the female parent. The First F_1 head was allowed to progress to anthesis and was checked for pollen production. If pollen was produced the head was bagged and allowed to self-fertilise and subsequent heads were then emasculated (as above) If the F_1 did not produce pollen the heads were pollinated without emasculation. Euploid wheat was used as to repeatedly pollinate the F_1s producing backcross one (BC_1) seed.

Crossing lines post BC_1

The same crossing technique was used for BC_1 and all subsequent crossed generations. Heads were emasculated (as above) and then pollinated with euploid wheat (as above) producing BC_2, BC_3 and BC_4 seed etc. Backcrossing continued until genotyping (chapters 4 and 5) showed single introgressions.

Self-fertilisation protocol

Plants to be self-fertilised were grown in glasshouse conditions as described above. Heads were covered with a glassine bags once they emerged from the flag leaf. After bagging, plants were left to grow until harvested (as above).

3.2.2 Analysis protocols

Germination Rates

Germination rate was calculated by dividing the number of successfully germinated seed in a generation, by the total number of seed sown. Germination percentage was calculated by multiplying the germination rate by 100. A wheat control germination rate was calculated from 210 paragon seed germinated in the same method as non-*in situ* seed, (see above).

$$\%\ germination\ = \frac{successful\ germination}{seed\ sown} \times\ 100$$

Fertility

Percentage of crossed heads to set seed was taken as a proxy for fertility as more accurate measurement was impractical. Fertility was calculated by dividing the number of crossed heads to successfully set seed by the total number of heads crossed.

$$\%\ fertility\ = \frac{seed\ setting\ crossed\ head}{total\ crossed\ heads} \times\ 100$$

Shrivelled seed

Visual inspection was used to count the number of shrivelled seed, see figure 3.6. Percentage shrivelled seed was calculated by dividing the number of shrivelled seed by the total number of seed. A wheat control was calculated from 2663 paragon seed.

$$\%\ Shrivelled\ seed\ = \frac{shrivelled\ \ seed}{total\ seed} \times 100$$

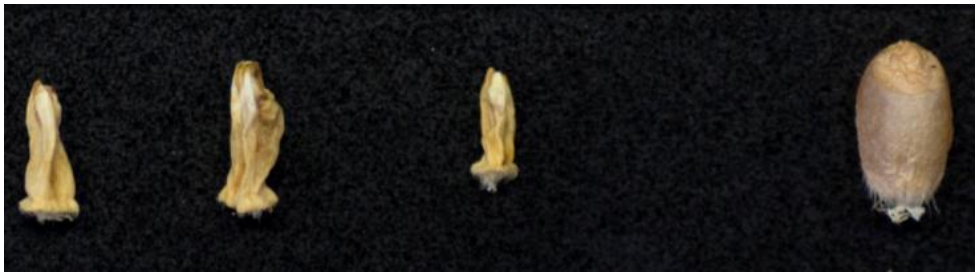


Figure 3.6 Pictures of seed produced from *S. cereale* crosses. The left three are shrivelled, while the seed on the right is a normal filled seed.

Seed per crossed head

Seed per crossed head was calculated by dividing the number of seed produced by the number of crossed heads.

$$Seed\ per\ crossed\ head\ = \frac{seed}{crossed\ heads}$$

Viable seed per cross

Viable seed per cross was calculated by multiplying the number of seed per cross by the germination rate.

$$viable\ seed\ per\ cross = seed\ per\ cross\ \times\ germination\ rate$$

3.3 41__Germplasm Results:

In total, over 49,200 seed have been produced from *S cereale*; 5,087 seed from 2469 crosses and over 44,200 seed via self-fertilisation. Table 3.1 shows a breakdown of seed produced according to accession, generation, and method of production.

Table 3.1: Total numbers of seed that produced. Each row is the method used to produce seed (crossing or self-fertilisation). Each column shows a generation used to produce seed.

Production	**Initial**	**F_1**	**BC_1**	**BC_2**	**BC_2F_1**	**BC_2F_2**	**BC_3**	**BC_3F_1**	**BC_4**
Cross	676	355	320	2643	-	-	1096	-	-
Self	n/a	-	-	827	18000	6000	1570	17000	807

All eight accessions were successfully used to produce F_1 seed. F_1 plants from *S. cereale* 390382 produced 352 seed. F_1 plants from *S. cereale* 107, 428373, Palazzo and 426170 each produced 1 BC_1 seed resulting in a bottle neck limiting the efficacy of these lines. Only accessions 390382 and 428373 progressed to BC_2, producing 252 and 68 seed respectively (table 3.2). For ease of analysis accessions have been grouped and studied as a whole unless otherwise stated.

Table 3.2 The number of F_1, BC_1 and BC_2 seed produced from each accession of *S. cereale*

Generation	Palazzo	Brasetto	107	Blanco	578092	390382	426170	428373
F_1	64	74	60	19	61	30	236	132
BC_1	1	-	1	-	-	352	1	1
BC_2	-	-	-	-	-	252	-	68

Germination Rates

The Germination rate of the F_1 interspecific hybrids and backcross generations 1, 2, 3, and 4 was 28.9%, 17.9%, 86.9%, 66.6%, and 86.4% respectively (Figure 3.5). The wheat control showed 92.3% germination. The BC_1 and F_1 seed both had low germination rates. The germination rates improved in the BC_2 seed and subsequent generations.

Fertility

As a proxy for fertility the % of crossed heads that produced seed was taken. The initial cross between rye and wheat *ph1* mutant had 41.6% fertile crossed heads. Crossed heads of the F_1 interspecific hybrids and backcross generations 1, 2, 3, 4 had 13.1%, 20.1%, 66.6%, 91.2%, 98.9% fertile crossed heads respectively (Figure 3.6). The initial cross was more fertile than the F_1 and BC_1. F_1 showed the lowest fertility. However, fertility then increased with each subsequent generation.

Shrivelled seed

When the F_1 seed produced was examined it was found that 24.2% was shrivelled while the BC_1 seed was 30.7% shrivelled. Backcross generations 2, 3 and 4 had 3.9%, 8.2% and 0.4% shrivelled seed respectively (Figure 3.7). The wheat control had 0.8% shrivelled seed

Seed per crossed head

The initial cross between rye and wheat *ph1* mutant produced 4.93 seed per crossed head. The F_1 interspecific hybrids and backcross generations 1, 2 and 3 produced 0.40, 0.80, 5.64 and 13.14 seed per crossed head respectively (Figure 3.8) There is no data for BC_4 cross per head as all BC_4 were self-fertilised.

Viable seed per cross

The initial cross between rye and *ph1* mutant wheat produced 1.42 viable seed per head. The F_1 interspecific hybrids and backcross generations 1, 2 and 3 produced 0.07, 0.68, 3.72, and 11.35 viable seed per head respectively (Figure 3.9).

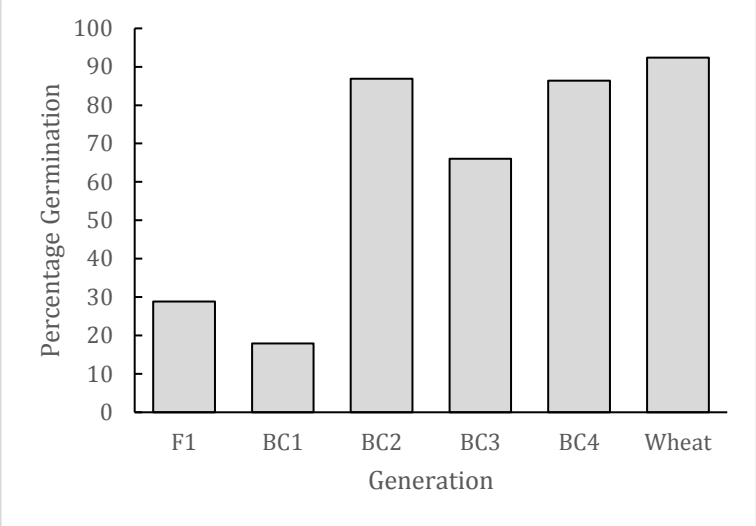


Figure 3.5 Germination rates of all accessions divided by generation. F_1 and BC_1 had low germination rates of 28.9% and 17.9%. Germination rates in the BC_2 and above was restored to wheat like levels. The F_1 generations germination rate was enhanced by embryo rescue

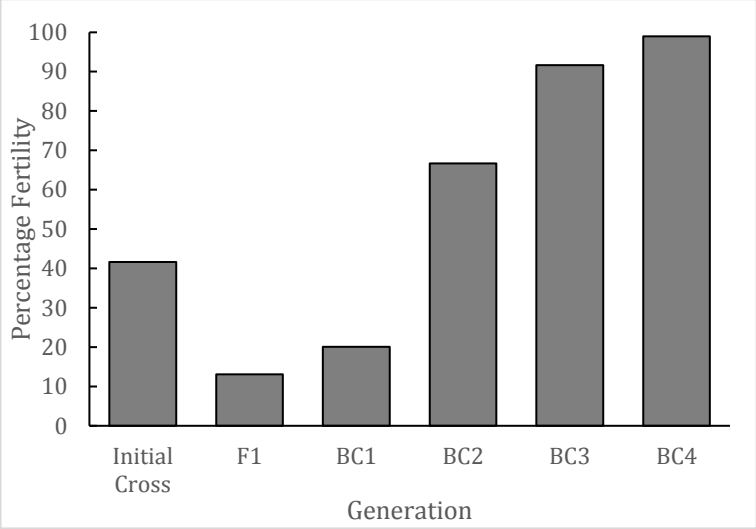


Figure 3.6 Fertility indicated by percentage of crossed heads that set seed. The initial cross had a high fertility of 41.6% compared to the fertility of the F_1 and BC_1, 13.1% and 20.1%. Fertility was 66.6% in the BC_2 and over 90% in BC_3 and BC_4

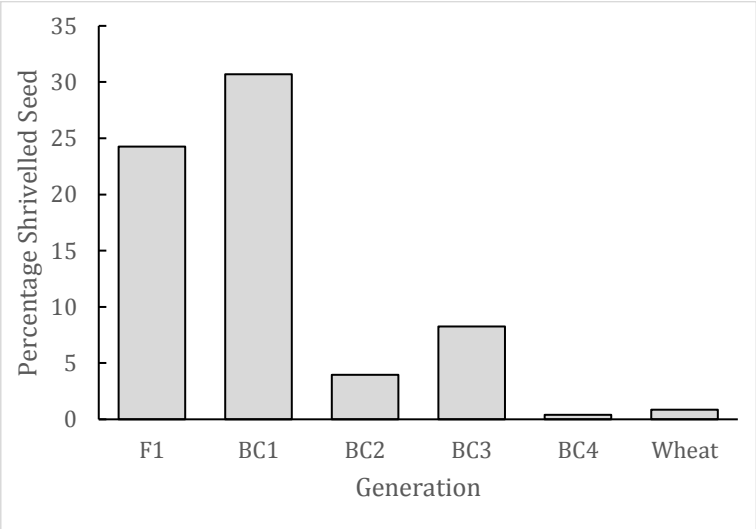


Figure 3.7 Percentage shrivelled seed produced. The F_1 and BC_1 seed was 24.2% and 30,7% shrivelled respectively. BC2 seed was 3.9% shrivelled. BC3 seed increased to 8.2% shrivelled likely due to a hessian fly infestation.

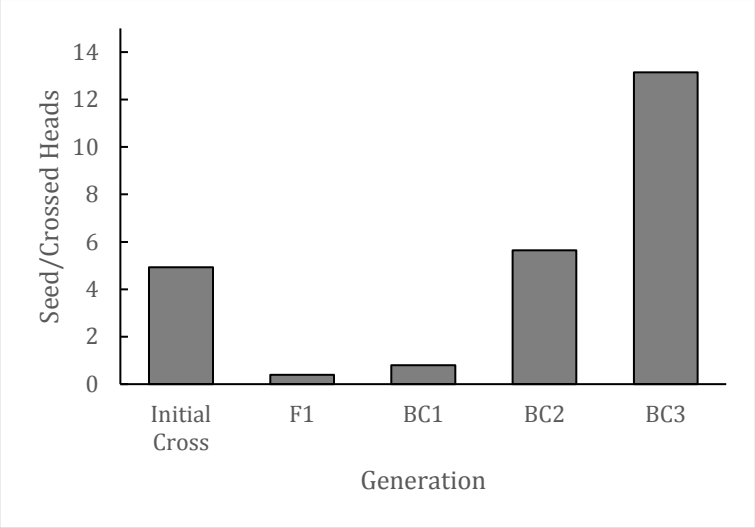


Figure 3.8 Number of seed per crossed head. F_1 and BC_1 produced less than 1 seed per head, 0.40 and 0.80 respectively. The initial cross and BC_2 produced 4.93 and 5.64 seed per head. Seed per head improved in the BC_3 to13.14

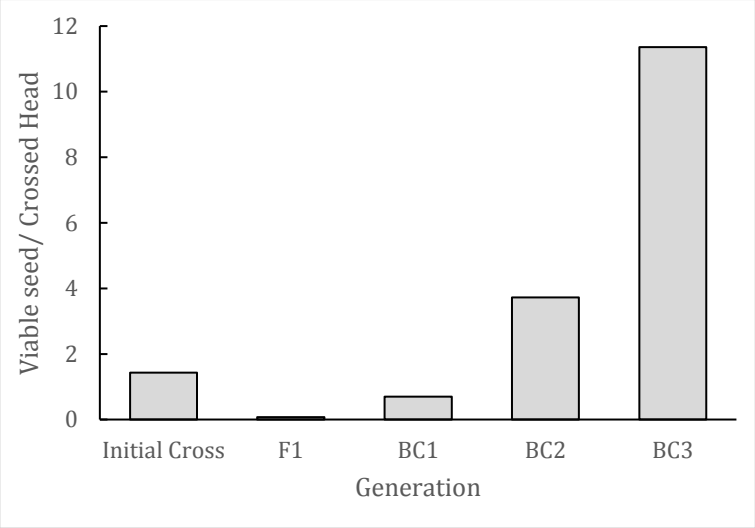


Figure 3.9, Number of viable seed per crossed head. Obtained by multiplying the seed produced per cross and the proportion of seeds that germinate. The F_1 produced the least viable seed per cross. With each successive back cross increasing the number of viable seed per cross

Overview

The initial cross yielded a large viable seed set compared to the F_1 and BC_1. generations. The majority of F_1 plants where obtained via embryo rescue which increased the number of viable seed produced in the initial cross.

The F_1 plants had low fertility and sterile anthers. Hence the F_1 could only be used as the female parent. To produce seed from the F_1 plants, large numbers of heads needed to be crossed producing low seed sets. The small

seed set of the F_1 plants was compounded because the BC_1 had the largest proportion of shrivelled seed, with many lacking an embryo. It was also observed that the F_1 plants were often unhealthy and a larger proportion of the F_1 plants died after germination but before reaching anthesis than in any other generation.

BC_1 plants showed a slight increase in both fertility and viable seed set than the F_1 plants but these were still low compared to both future generations and the initial cross. BC_1 plants also required many crossed heads to produce a low seed set. It was observed that the BC_1 plants were also often unhealthy and more plants died before anthesis than in subsequent generations.

BC_2 seed had low amounts of shrivelled seed and a 86.9% germination that was close to the wheats 92.3% germination. BC_2 plants showed intermediate fertility between the BC_1 plants and subsequent generations. The BC_3 seed had lower germination rates than the BC_2 seed. It is likely, however, that the BC_2 plants reduced fertility, together with the poor quality of the BC_3 seeds, have been negatively distorted by a Hessian fly infestation, which occurred during the time that the majority of BC_2 plants were in the glasshouse (seed quality refers to the germination rate of the seed and the proportion of shrivelled seed. Seed quality should not be confused with grain quality that refers to end use characteristics).

In the BC_3 generations and above, fertility and seed set was restored to wheat like levels. Improved fertility reduced the number of crosses necessary per plant as more were successful and each cross produced a large seed number.

3.4 Germplasm Discussion

This programme forgoes a more traditional targeted approach to gene transfer in favour of a 'shotgun' method intended to transfer the whole rye genome into wheat. The rationale of this approach was that the introgression of a targeted trait is limiting and may prohibit the capture of a range of other traits, which may not be foreseeable.

Importance of the F1

The F_1 population is key in producing successful introgressions, the reasons are twofold. Firstly, the F_1 contain the haploid genomes A, B and D of wheat and R of rye. Each genome is haploid and thus there are no true homologues. Therefore, homoeologues are forced to pair. Secondly, if the F_1 hybrid was produced from euploid wheat homoeologous chromosome pairing is normally inhibited because of the presence of an active *Ph1* locus (Moore 2015). In the crossing scheme employed here, the F_1 hybrids were produced from wheat lines with the *Ph1* locus deleted. Therefore, In the meiosis of the F_1 hybrid lacking *Ph1,* it was hypothesised that recombination could occur between the wheat and rye genomes, resulting in gametes with chromosomes containing wheat rye recombinants. The wheat-rye recombinant chromosomes were then backcrossed into wheat to produce introgression lines. The recombination occurring at meiosis of the F_1 hybrid is key as if there is no exchange of chromatin between the wheat and rye genomes at this step, recombined rye chromatin cannot be captured in subsequent crosses. The effectiveness of the introgression production is discussed in subsequent chapters.

Seed production

The initial F_1 population, though key to the program, was comparatively easy to produce. The initial cross was less fertile and produced less seed than a wild wheat plant, but still produced multiple seed per head, which was higher than the F_1 and BC_1 generations. The increased seed production may

be because in the initial cross the female parent is a fertile and crossable variety of wheat that is fitter/more fertile than the F_1 and BC_1. Seed produced was of poor quality but through extensive embryo rescue it was possible to increase germination rates.

Seed production from the F_1 and BC_1 generations proved to be challenging. Small amounts of poor quality seed were obtained. F_1 and BC_1 plants produced no seed from self-fertilisation. Two possible reasons may explain the poor seed production: Firstly, unstable chromosome numbers and the lack of true homologues may inhibit meiosis and therefore stop gametogenesis, stopping seeds from forming. Secondly, the high proportion of rye chromatin and a lack of some wheat chromatin resulting in an inferior combination of genes that may lack some necessary genes therefore causing weak and unhealthy plants that are unable to produce large seed sets.

It was hypothesized that seed production and germination rates, after F_1 production, would increase with each successive backcross due to a return to more stable chromosome numbers and the restoration of wheat chromatin. This hypothesis was accurate for fertility, the number of seed per crossed head and viable seed per head, as each metric was shown to increase with successive back crosses. Germination rates were expected to increase with each generation and the number of shrivelled seed was expected to decrease. Broadly this prediction held true except for the BC_3 seed that bucked the trend with lower germination rates and a higher proportion of shrivelled seed than from BC_2 seed. This unexpected result is most likely due to a hessian fly infestation that occurred while the majority of BC_2 plants were in the glasshouse, negatively effecting the plants health and the quality of seed they produced.

During the early generations, i.e. F_1, BC_1, and to a lesser extent BC_2, there was a chance that captured introgressions could be lost due to an inability to produce viable offspring. To counteract this, it was necessary to produce

as many seed as possible from each generation. Therefore, as many plants as possible were progressed through the crossing scheme, including plants that may have contained the same introgressed region. Hence, in the early generations a large amount of labour in the form of crossing and embryo rescue was needed for a small population of plants with low seed numbers.

After the BC_2 generation, fertility and seed quality was partially restored, compared to wheat, with a higher proportion of successful crosses and more seed being produced per cross. Hence, crossing could be less extensive and embryo rescue was not necessary. In the later generations emphasis changed from producing seed from small numbers of plants to managing large populations. Large populations were necessary at the higher back crosses to maintain maximum coverage of the rye genome, because individual plants contain less rye chromatin than during earlier generations.

Plant numbers greatly increased once the 'self-fertilisation' stage began because of the need to check for chromosome segregation. Theoretically, in the absence of other factors such as meiotic drive, a quarter of the self-fertilized seed will lose an introgression, half will remain heterozygous and a further quarter will contain the targeted homozygous introgression. Plants that reach the 'self-fertilisation' stage had similar fertility and germination rates as wheat plants due to a restoration of the wheat genome and comparatively less rye chromatin.

Observations

During the backcrossing stage every plant was a genetic individual, meaning that any observation of phenotype was unreliable because not replicates were available and the plants genetic composition changes between generations. Replicates were only obtained at the end of the crossing scheme once selection for homozygosity was complete and have not yet undergone any phenotyping trails. The above point not withstanding there were some noticeable differences between the back-cross generations. The

F_1 plants produced large numbers of tillers, in some extreme cases over 40. For comparison the rye accession 390382 produced three tillers per plant in a Polish field trial (Banaszczyk *et al.* 1992) and 3-5 tillers per plant when grown for the initial cross (data not shown). Tiller numbers steadily decreased with each successive back cross. The increased tillers in the early generations was likely linked to the plants' reduced fertility with energy being used to produce more tillers instead of being used in seed production and filling. The F_1 also showed morphological traits resembling rye. For example, some plants developed awns and had thin spikes and seed compared to Paragon. The frequency of such traits seemed to decrease with successive back-crossing. Other possibly interesting morphological traits were produced that may warrant future study such as a broad range of plant heights and spike morphology (figure 3.10) including branching spikes in the BC_2 generation (data not shown).

Figure 3.10 Three images of BC_1 spikes with differing morphology

Seed generation conclusion

In summary the crossing scheme has been shown to be successful in producing large numbers of plants with different amounts of rye germplasm. This crossing scheme faced two major challenges. Firstly, the low fertility and the poor seed numbers in the early stages of the crossing scheme, i.e. the F_1 and BC_1 generations. This initial challenge can be overcome by increasing the number of crosses made and the use of embryo rescue techniques. Hence, the initial stages of the program were labour intensive. The second challenge was the large number of plants, particularly in later generations (BC_3 and above as well as the self-fertilizing populations) and subsequent genotyping large numbers of individuals. The number of plants to be genotyped demanded the use of high-throughput genotyping methods, discussed in chapters 4 and 5.

4 Cytogenetics Investigation

4.1 Cytogenetics Introduction

Wild relative introgressions have been traditionally investigated using cytogenetic techniques. Much of the early work identifying wild relative introgressions was undertaken using C-banding. Rye heterochromatin was first identified and karyotyped using C-banding in the early 1970s (Sarma *et al.* 1973; Gill and Kimber 1974b). C-banding was first used to identify wheat-rye introgressions by Lukaszewski and Gustafson (1983). FISH and GISH has replaced C-banding in most modern research.

Various sizes of DNA can be used to create a *in situ* hybridisation (ISH) probes ranging from small oligonucleotide sequences (Tang *et al.* 2014), to the most common probes of 100-600bps (FISH) and ranging all the way to total genomic DNA being used as a probe in GISH. ISH is an exceptionally versatile and informative technique and its uses range from as a diagnostic tool in human pathology (McNicol and Farquharson 1997) to FISH probes that can identify wheat centromeres,[CCSI (Aragón-Alcaide *et al.* 1996)] and rye centromeres, [pAWRC.1 (Francki 2001)]. The data gathered varies depending on the chosen DNA sequence. The repetitive sequences pAs1, pSc119.2 and pTa71 are some of the most commonly used FISH probes and can characterise wheat, rye, and wheat-rye hybrids (Bedbrook *et al.* 1980; McIntyre *et al.* 1990; Nagaki *et al.* 1995; Cuadrado *et al.* 1997; Contento *et al.* 2005; Fujisawa *et al.* 2006; Fu *et al.* 2013; Hao *et al.* 2013).

FISH using repetitive sequences is limited as a tool for detecting introgression lines as the resolution is too low to detect small introgressions (if a small segment does not contain a hybridisation site it cannot be detected). Total genomic DNA (GISH) has a few key advantages over repetitive sequences (FISH) when used as a probe. Firstly, a genomic probe identifies the presence of an introgressions irrespective of sequence. Secondly, compared to FISH, analysis of GISH is faster, easier to interpret

and does not require a pre-existing karyotype. However, GISH is unable to distinguish between individual chromosomes which is possible using FISH.

This work aims to use genomic *In situ* hybridisation to (i) identify wheat-rye introgressions, (ii) validate the molecular genotyping in chapter 5 (iii) using multi-colour GISH, identify the wheat genome containing each introgression. Due to the confusion in terminology between what is an introgression, recombinant and translocations all transfer rye chromatin into a wheat background are referred to as introgressions until the discussion where sufficient data is available to discriminate between the different types of captured rye chromatin.

(i) Identification and Validation

GISH is commonly used to identity wild relative germplasm within important crop species including rice, cotton and wheat (Schwarzacher *et al.* 1992; Apisitwanich 1999; Wang *et al.* 2018) as it can effectively identify all but the smallest introgressions without bias. Recent studies have used GISH to characterise new 1BL:1RS translocation lines with positive effects on yield and stripe rust resistance (Qi *et al.* 2016).

(ii) Genotyping Validation

Cytogenetic techniques can be used to validate molecular genotyping. Previous work by the BBRSC Wheat Research Centre, based at the University of Nottingham, has effectively used GISH to validate molecular genotyping techniques for *Am. muticum* (King et al. 2017) and *Th. bessarabicum* (Grewal et al. 2017a) and thus the cytogenetic results described in this chapter are integral to chapter five.

(iii)Multi-colour GISH

Once introgression lines containing a recombinant/translocation have been obtained, multiple-colour GISH will be used on those lines to visualise the wheat genome that the rye chromatin has been captured in.

4.2 Cytogenetics Materials and Methods

Cytogenetics Germplasm

The seed used was generated as described in chapter 3. A complete set of wheat/rye (Imperial) addition lines obtained from the Germplasm Resource Unit (GRU) at the John Innes Centre (chapter 2). Seed was germinated and grown as described in 3.2.

Slide preparation

Fixing root cells at metaphase

Metaphase spread slide preparations were made using the same method for single and multi-colour GISH. Roots were collected from plants germinated on filter paper in petri dishes, one to two days after germination. The distal 5mm - 30mm was taken using fine nosed forceps insuring the root tip was collected. Roots from embryo rescued seed were collected from plants once they were transferred to soil, with the youngest roots visible being selected.

Roots were immediately placed in 0.5 ml micro centrifuge tubes and sprayed with water to avoid dehydration. The lid of the micro centrifuge tube was punctured, closed and then placed in a pressure chamber. Root tips were treated with nitrous oxide at 10 bar for ~2hours. After nitrous treatment, root tips were fixed with 90% acetic acid for 10 minutes and then washed a minimum of three times with deionised water to completely remove the acetic acid. Root tips were transferred to new microcentrifuge tubes and filled with deionised water. For short term storage (less than one month) roots were kept in a fridge at 4°C, for longer term storage, roots were kept in a freezer at -20°C. Originally root tips were stored in 70% ethanol instead of deionised water, but this practise resulted in lower quality slide preparations.

Enzyme digestion

After the fixation treatment, root tips were cut away from the remaining root. Approximately 2mm of the bright white region of root tip was taken using a razor blade and a black tile. Root tips were digested in 20 µl of 2% cellulase onozuka R-10 and 1% pectolyase Y23 solution (Yakult Pharmaceutical, Tokyo) at 37°C, for 47 - 55 minutes (depending on root tip size) for root tips collected from petri dishes and 55 - 65 minutes for root tips collected from soil. After enzyme digestion, root tips were immediately cooled to stop the reaction, washed 3 times in 70% ethanol and suspended in 100 µl 70% ethanol.

Slide production

The root tips suspended in 100 µl of 70% ethanol were gently crushed against the side of the microcentrifuge tube with a dissection needle. Samples were centrifuged at 5,000 rpm for 2 minutes, the ethanol removed, and the tubes left to air dry briefly. 16-30ul of 100% acetic acid was added and the tubes agitated gently to produce a cell suspension and left on ice for a minimum of 30 minutes. Glass microscope slides were then prepared in a moistened tissue lined box. 7µl of the acetic acid cell suspension was then carefully pipetted onto the microscope slides from a height of ~70mm. Microscope slides were left to dry in a moist environment. Slides were checked for quality with phase contrast using a Leica DM 1000 LED microscope.

Probing procedures

Genomic DNA Extraction

Genomic DNA was extracted using a modified version of the CTAB method used in King *et al.* (2017)(section 2.2 page 36-37)

Blocking DNA

Blocking DNA for GISH was produced from complete genomic DNA, extracted as described above. Complete genomic DNA was fragmented by heating to 100°C in a heat block for 20 minutes.

Probe production: Nick translation

Nick translation was used to produce probes from plasmid DNA or genomic DNA by incorporating fluorescently labelled dNTPs. The reaction mixture (table 4.1) was mixed and then incubated at 16°C for 2 hours in the dark.

Table 4.1: Reaction solutions for nick translation for both genomic and plasmid DNA and PCR products. Deionised water, dH_20 was used to insure the total reaction mixture was 20µl

Reagent	Genomic DNA	Plasmid DNA	PCR Product
Plasmid DNA	-	2µg	-
Genomic DNA	2µg	-	-
PCR product	-	-	1µg
10X Nick-translation buffer	2 µl	2 µl	2 µl
Non- Labelled dNTPs (2mM each, mixed)	2 µl	2 µl	2 µl
Labelled dNTPs (1mM)	0.5 µl	0.5 µl	0.5 µl
DNA polymerase 1 (10U/µl)	5 µl	4 µl	4 µl
DNase (100 mU/µl)	0.8 µl	0.5 µl	0.5 µl
dH_20		(n) to total 20 µl	

Probe production: precipitation

160 µl (eight times of the nick translation reaction volume) of 140 ng/µl salmon sperm DNA working solution (appendix 1) and 500 µl (25 times of the nick translation reaction volume) of precipitation solution (appendix 1) were added to the nick translation solution and incubated overnight at -20°C. The resulting solution was spun at 12,000 rpm for 30 minutes at 4°C to pellet the probe. The probe pellet was washed three times with 70%

ethanol and the supernatant removed. The probe pellet was then resuspended in 20 µl of 2xSSC + 1xTE (appendix) and stored in the dark at -20°C.

Single colour GISH

For single colour GISH, rye genomic DNA (accession 390382) was used as probe and labelled with Alexa Fluor 488 [green] (www.thermofisher.com). Chinese Spring genomic DNA was used as blocking DNA. The probing mixture for single colour GISH is shown in table 4.2. Slides were probed as described below.

Table 4.2 The solution prepared to probe one slide with Single Colour GISH. The probes excitation wave length (nm) is given in bold along with its nearest colour

Single Colour GISH	
Probe	
Rye genomic DNA ***GREEN 488***	*1 µl/0.1 µg*
Blocking DNA	
Chinese Spring ***BLUE 358 (DAPI counterstain)***	*2.5 µg rye probe*
2xSSC 1xTE	*to total 10 µl*

Multi-colour GISH

Each Slide was probed with three probes, described above. Rye Genomic DNA (accession 390382) was labelled with 14-dUTP ChromaTide(r) Alexa Fluor(r) 546 nm (www.thermofisher.com), [gold]. *T. uratu* genomic DNA with 5-dUTP ChromaTide(r) Alexa Fluor(r) 488 nm (www.thermofisher.com), [green] and *Ae. tauschii* genomic DNA was labelled with 5-dUTP ChromaTide(r) Alexa Fluor(r) 594nm (www.thermofisher.com), [red]. *Ae. speltoides* genomic DNA was used as blocking DNA and therefore is visible with the DAPI counterstain [blue]. Table 4.3 shows the probing mixture used to probe one multi-colour GISH slide. Slides were probed as described below.

Table 4.3 The solution prepared to probe one slide with Multi-Colour GISH. The probes excitation wavelength (nm) is given in bold along with its nearest colour.

Multi-colour GISH	
Probes	
Rye **GOLD 546**	1 µl/1µg
T. uratu **GREEN 488**	1 µl/1µg
Ae. tauschii **RED 594**	1 µl/1µg
Blocking DNA	
Ae. speltodies **BLUE (DAPI counterstain)**	x 2.5 µg
2xSSC 1xTE	to total 10 µl

Probing protocol

Slides were probed with the same method for single and multi-colour GISH as well as FISH. Slides were UV crossed linked with 0.125 joules twice. For all steps post crosslinking, slides were kept in the dark. 10 µl of probe solution, depending on the GISH/FISH method chosen (chapter 4.2), was pipetted onto each slide. Slides were covered with plastic cover slips avoiding air bubbles. The slide was denatured at 75°C in a moist environment for 5 minutes and then incubated over night at 55°C in a water bath in a moist environment to hybridise the probe. After hybridisation the plastic cover slip was removed by immersing the slide in 2xSSC (appendix 1). A drop of Vectorshield mounting medium with 4,6-diamidino-2-phenylindole (DAPI) (appendix 1) was pipetted onto each slide and then a glass cover slip was carefully applied avoiding air bubbles. The slides were then ready to be visualised using a MetaSystems Coolcube 1m CCD camera. Further, slide analysis was carried out using Meta Systems ISIS and Metafer software (Metasystems, Altlussheim, Germany). This system enabled the fully automated capture of high- and lowpower fluorescent images of root tip metaphase spreads. Slides with root tip preparations were automatically scanned and the images downloaded for analysis.

Identification of introgressions and genotyping validation

Due to logistical and procedural constraints, only a proportion of the introgression lines produced were genotyped using cytogenetic techniques. Metaphase chromosome spreads were prepared from the introgression lines produced in chapter 3. Slides were initially probed and imaged with single colour GISH, described above. Lines that were shown to contain introgressions where then observed using multi-colour GISH, described above. The data obtained from single colour GISH was used to validate the molecular genotyping discussed in chapter 5.

4.3 Cytogenetics Results

Single Colour GISH

To identify wheat rye introgressions root tips from 306 plants were analysed with single colour GISH. Root tips were taken from the BC_1, BC_2, BC_2F_1, BC_2F_2, BC_2F_3, BC_3, BC_3F_1, BC_3F_2, BC_4, BC_4F_1, and BC_4F_2 generations. Rye chromosomes observed were classified into three groups: whole chromosomes, chromosome fragments/aberrations (mainly telocentric chromosomes) and translocations/ recombinants. Telocentric chromosomes are chromosome with the centromere at the terminal end of the chromosome; the telocentric chromosomes described are the formed by a acrocentric chromosome fragmenting at the centromere and the loss of one chromosome arm while retaining the second chromosome arm and the centromere. The translocations described are majorly centric fusions formed by the breaking of two chromosomes at the centromere and the fusion of the whole chromosome arms from the two distinct chromosomes, i.e a whole wheat long chromosome arm fusing with a whole rye short arm. Figures 4.1 show examples of the different classes of introgressions. Figure 4.2 shows a selection of rye introgression captured in a wheat chromosome Table 4.4 shows a breakdown of the introgressions produced by generation. Table 4.5 shows the percentage of each introgression type per generation.

Table 4.4. A summary of lines observed with single colour GISH. The table shows the number of plants observed per generation and the number of chromosomes with rye chromatin sub-divided into whole rye chromosomes, chromosomal aberrations, and translocations/recombinant chromosomes.

	BC_1	BC_2	BC_2F_1	BC_2F_2	BC_2F_3	BC_3	BC_3F_1	BC_3F_2	BC_4	BC_4F_1	BC_4F_2
Lines Genotyped	1	55	16	8	12	86	46	21	34	19	1
Whole	6	151	20	7	16	111	29	5	16	8	0
Aberrations	0	2	2	0	0	7	2	2	2	1	0
Translocations / Recombinants	0	2	4	4	11	4	5	0	3	1	0
Mean number of introgressions	6.00	2.85	1.63	1.37	2.25	1.41	0.78	0.33	0.61	0.52	0

Table 4.5. The percentage of each introgression type by generation

	BC_1	BC_2	BC_2F_1	BC_2F_2	BC_2F_3	BC_3	BC_3F_1	BC_3F_2	BC_4	BC_4F_1
Whole	100.00	97.42	76.92	63.64	59.26	90.98	80.56	71.43	76.19	80.00
Aberrations	0.00	1.29	7.69	0.00	0.00	5.74	5.56	28.57	9.52	10.00
Translocations / Recombinants	0.00	1.29	15.38	36.36	40.74	3.28	13.89	0.00	14.29	10.00

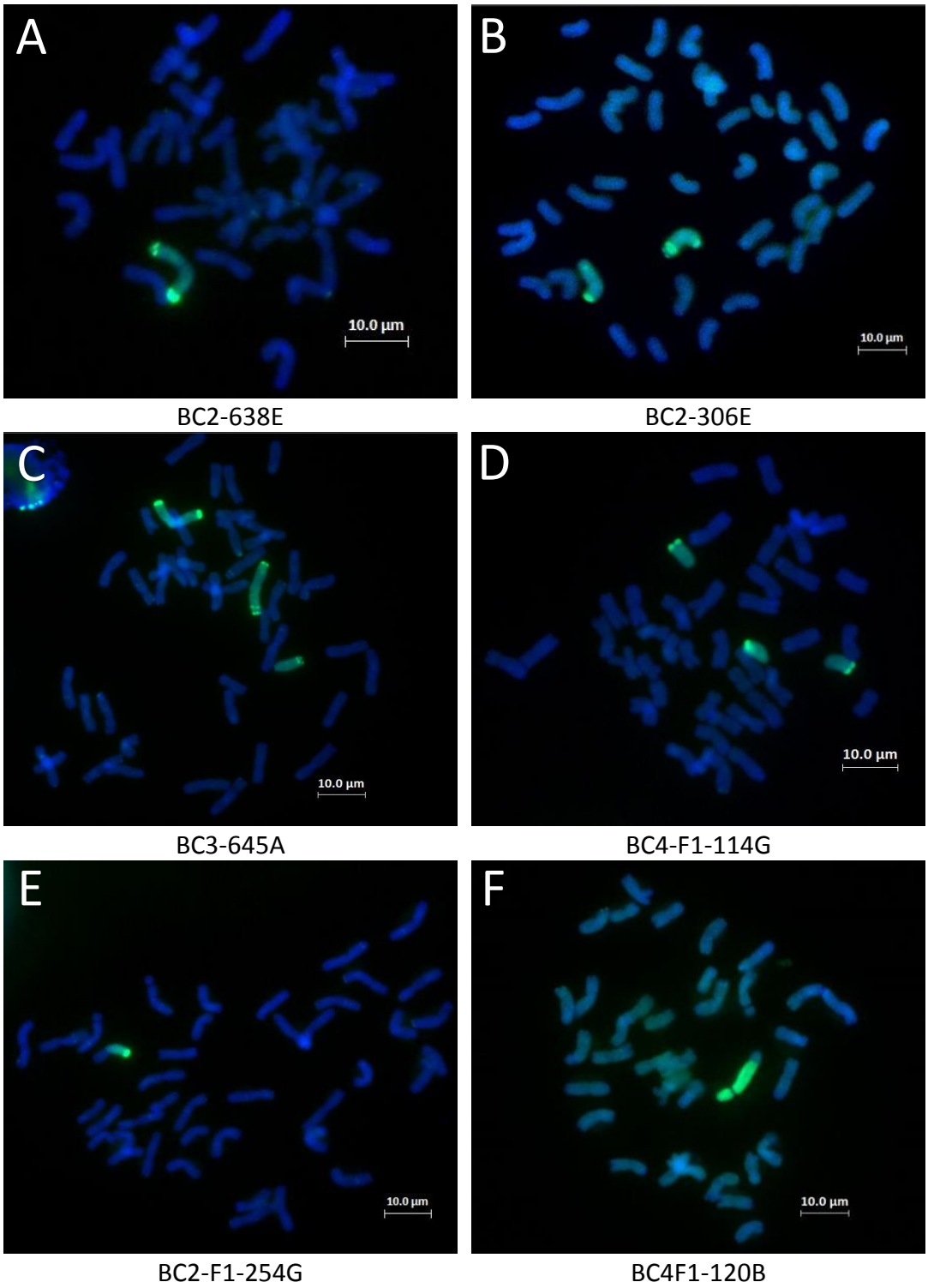


Figure 4.1. GISH images of six types of introgression lines. Rye germplasm is shown with green using complete genomic Rye DNA as probe, wheat germplasm is blue, counterstained with DAPI. BC2-638E (A) shows a single whole chromosome introgression. BC2-306E (B) shows two introgressed whole rye chromosomes. BC3-645A (C) shows two introgressed whole rye chromosomes and one telocentric chromosome. BC4-F1-114G (D) shows three rye chromosome aberrations/telocentric chromosomes. BC2-F1-254G (E) shows a wheat-rye translocation (centric fusion). BC4F1-120B (F) is a wheat-rye recombinant

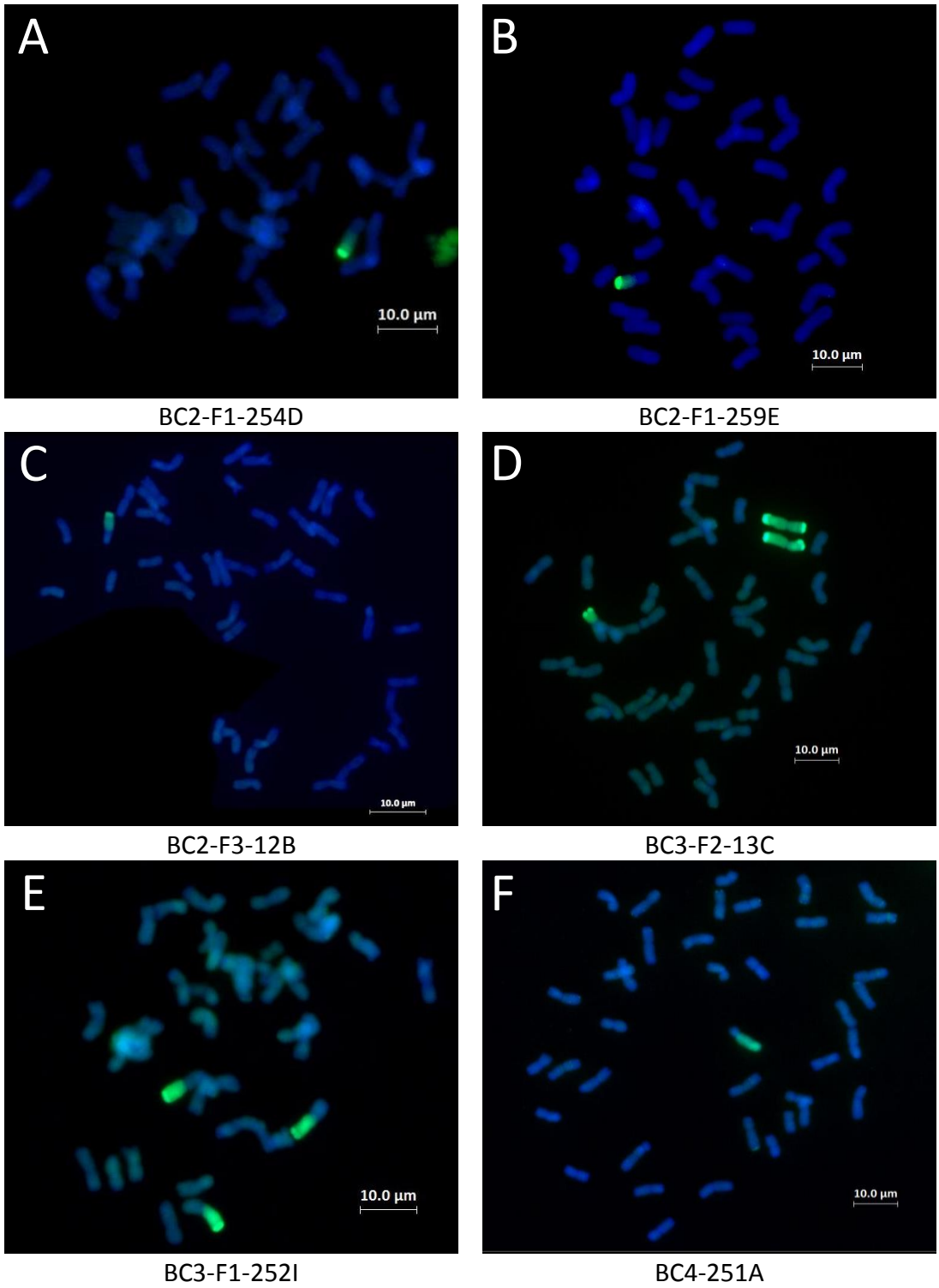


Figure 4.2. GISH images of six wheat-rye recombinants/translocations. A-E are most likely the product of centric fusion and F is a wheat-rye recombinant. Rye germplasm is stained green using complete rye genomic DNA as a probe. Wheat germplasm is blue, counterstained with DAPI. (D)BC3-F2-13C also shows two whole chromosomes additions.

BC_1

A single BC_1 was analysed with GISH and showed 6 complete rye chromosomes.

BC_2

55 BC_2 lines were analysed with GISH. The mean number of introgressions per plant was 2.85. The majority (97.4%) were whole chromosomes with aberrations and translocations/recombinants each accounting for 1.3% of the total introgressions.

BC_3 and above

After BC_2, introgressions were positively selected and therefore the results are skewed in favour of the translocations/recombinants. The percentage of whole chromosome introgressions, chromosome aberrations and translocations/recombinants are shown in table 4.5.

The mean number of introgressions reduces with each successive generation including self-fertilisation and crossing. The proportion of introgressions shifts towards recombinants and translocations with each generation.

Multi-Colour GISH

After identification with single colour GISH, lines containing introgressions of interest were then probed with multi-colour GISH, to determine which wheat genome has recombined. 17 lines were analysed with multicolour GISH. Figure 4.3. shows a selection of multi-colour GISH images.

Analysis of the 17 lines showed no introgressions in the A genome, eight introgressions in the B genome, seven introgressions in the D genome, and three introgressions where fragments of rye chromosome not attached to a wheat chromosome. The number of introgressions was more than the total number of lines as one line had multiple different introgressions. Some of

the lines visualised with GISH had been previously self-fertilised this can cause a problem when analysing the results as if an introgression is visually similar it is impossible to distinguish between to separate introgression or one introgression that has gone homozygous.

Multi-colour GISH also showed recombination between the wheat genomes. Three lines showed recombination between the A and D genomes and one line showed recombination between the B and D genomes.

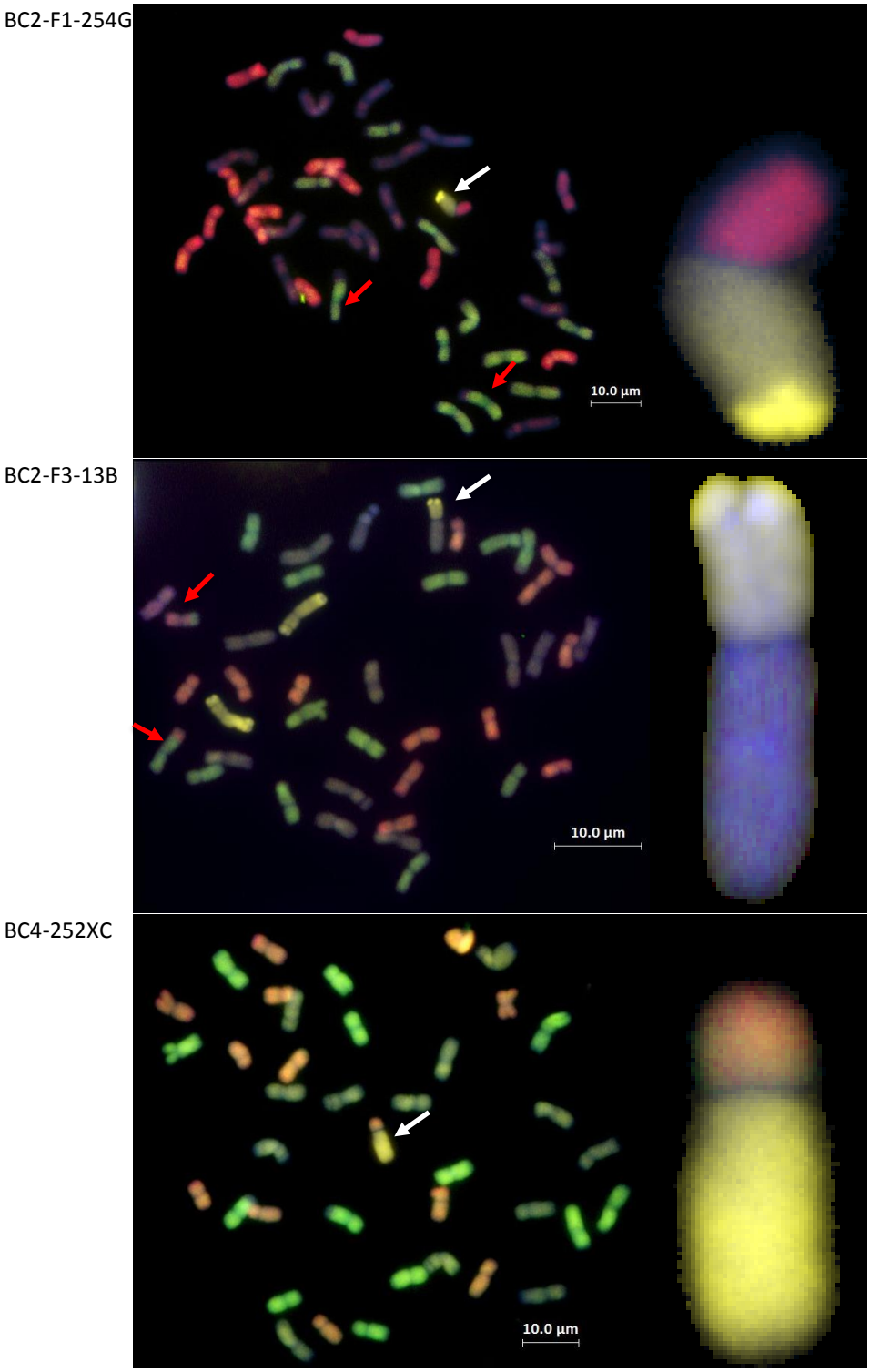


Figure 4.3. Three multi-coloured GISH images showing three rye introgression lines. The A genome has been stained green with a *T. urartu* probe. The D genome has been stained red with an *Ae. Tauschii* probe. The B genome has not been probed and is shown in blue with a DAPI counterstain. The *S. cereale* introgressions have been stained yellow with an *S. cereale* probe. The left images are complete metaphase spreads, and the right images are the chromosome containing the introgression. BC2-F1-254G shows a rye centric fusion into the D genome. BC2-F3-13B shows a rye centric fusion with the B genome and recombination between the A and D genomes. BC4-252XC shows a rye recombinant into the D genome and is from the same parental plant as BC4-251A shown in Fig 4.2F. White arrows indicate wheat-rye introgressions. Red arrows indicate recombinants between wheat genomes

4.4 Cytogenetics Discussion

Comparison of generations

The initial F_1 and BC_1 generation were, in general, not investigated using cytogenetic techniques due to logistical constraints (the lines were developed before the start of this PhD and before the cytogenetic investigation had begun). The F_1s that were imaged with single colour GISH showed seven rye chromosomes and 21 wheat chromosomes as expected. Observation of the F_1s was only used to confirm the F_1s were genuine hybrids. Recombination only occurs during meiosis in the production of gametes in the F_1s. and therefore introgressions would not be present in these plants. Only a single BC_1 was GISHed and therefore selection for introgressions was not possible at this stage. Where possible, the BC_1 stage would be the most effective stage to observe and select introgressions as recombination will have occurred and the number of plants is still low.

The BC_2 generation is the only generation with a large number of GISHed lines without selection in the parents and are therefore the most useful generation to determine the ratios of each type of introgression produced. For generations following BC_2 selection had begun and therefore the results are skewed in favour of more recombinants/translocations. In the BC_2 generation, the majority of introgressions were whole chromosome with a only a small proportion of introgressions being the recombinants/translocations that the breeding scheme aimed to produce. The proportion of recombinants was low in respect to both the number of recombinants per line and the number of recombinants per introgressed whole chromosomes when compared to similar breeding schemes using different wild relative donors (Grewal *et al.* 2017b; King *et al.* 2017, Baker 2018), The low recombination rate indicates the presence of a further barrier beyond *Ph1.* Possible reasons for this barrier and further ways of

inducing recombination between wheat and rye are discussed in detail in chapter 6.

Size of Introgression

Of the wheat-rye recombinants/translocations visualised by GISH the minimum size was equivalent to a short chromosome arm. With the exclusion of one larger recombinant, all the recombinants/translocations, visualised by GISH were the size of whole chromosome arms. The proportion of introgressions which where the size of whole chromosome arms and the presence of chromosome aberrations that were the same size as chromosome arms was much higher than other sizes of introgressions. This likely due to the fact these introgressions were to be the result of chromosome fragmentation, caused by the stress placed on the plants genomes through the crossing scheme, the fragmented chromosomes from wheat and rye would then join producing translocated chromosomes, known as centric fusions.

Recombination between wheat genomes

From the 17 lines that were analysed with multi-colour GISH, four showed recombination between the wheat genomes. The selection within the breeding scheme and the choice of lines to multi-colour GISH was irrespective of possible recombination between wheats own genomes and therefore can be thought of as a random selection. Though a small sample size, 23.5% of lines showed recombination between the wheat genomes. The presence of more recombination between the wheat genomes compared to wheat-rye recombination confirms that the removal of *Ph1* has facilitated homoeologous recombination between the relatively similar wheat genomes but has not enabled homoeologous recombination between wheat and the more distantly related rye genome. Interestingly, recombination has occurred between the A and D genomes and the B and D genomes but not between the A and B genomes. This may be due to the

fact that the D genome is more closely related to the A and B genomes than the A and B genomes are to each other (Marcussen *et al.* 2014).

Limitations of a cytogenetic approach

Cytogenetics has provided valuable information on the presence and type of introgression produced and proved to be effective in confirming the molecular genotyping (chapter 5). The information gained from GISH is limited in the following ways. Firstly, GISH provides an estimation of the physical size of introgressions but does not provide data about genetic distances. Genetic distances provide information about rates of recombination and would thus indicate if an introgressed regions is likely to be broken apart and separated. Secondly, single colour GISH gives no information about the position of an introgression and although multi-colour GISH provides information about which wheat genome an introgression is located in, it does not show any further detail about the location of an introgression such as which homoeologous group it belongs to.

The number of lines it is feasible to observe using cytogenetic techniques is limiting because the process is labour intensive, time consuming per line and does not scale effectively. The cytogenetic method can be thought of as two distinct phases, first the slide and probe preparation and secondly the microscope analysis. Developments in microscopy have increased the speed, through-put, and image quality. For example, during this project we upgraded from a conventional manual microscope (Leica Microscope DM5500B) to ZEISS Axio Imager.Z2 using Metasystems software which is an automated, mechanised microscope that uses machine learning and image recognition software to find and image metaphase chromosome spreads.

The upgraded microscope system has enabled two key improvements. First, an increased throughput, 5-20 slides an hour using the automated Zeiss microscope and Metasystems software as compared to only three slides per

hour using the manual microscope. The increased slide number is compounded by the fact that the number of metaphases imaged per slide is also higher when using the Metasystems software compared to the number of images using a manual microscope. Secondly, modern microscopes can handle more phytochromes, enabling multi-colour GISH with four colours to be achieved in a single step, instead of a two-step process with the slide first being stained with a probe for an introgression and then bleached and stained again to probe with the wheat genomes. The main disadvantage of using the automated Zeiss microscope and Metasystems software is the need for more stringent slide preparation as differences between slides can be detrimental to the automated procedure and thus the system is less effective with poor quality slides. The automated microscope greatly improved throughput but moved the limiting factor away from the microscopy to slide preparation which is time consuming and requires a high technical skill level to obtain consistent, high-quality slides.

Observations of the cytogenetic technique

The following observations are anecdotal and therefore would need to be tested to be validated.

Slide production was key in producing high quality images. Root tips collected and nitrous oxide treated between 8:00 am and 11:00 am produced better quality slides than root tips collected later in the day. This increase in quality may be due to increased growth in the mornings due to the seedlings circadian rhythm (de Montaigu *et al.* 2010) resulting in more cells actively dividing while undergoing the nitrous oxide treatment, and therefore more metaphase spreads in the final preparations. Although slides were successfully prepared from both root tips a few days old in Petri dishes and from root tips collected from older plants in the soil, it was more difficult to successfully produce slides from older roots. This was due to a couple of reasons. Root cells were less likely to be actively dividing and therefore had less cells at metaphase. The roots were also more variable

and usually larger, with thicker cell walls and therefore the correct digestion of the cell wall was more challenging causing the quality of preparations to be more variable.

The initial root tips were stored, after nitrous oxide treatment, in 70% ethanol at -20˚C. The technique was later altered to storage in water at -20˚C as this resulted in higher quality slides and limited loss of quality after 6 months storage. This was likely due to the ethanol altering stored cells proteins or cell membrane (Eltoum *et al.* 2001)

When probing rye, if the ratio of blocking DNA was not correct, the genomic rye probe would only bind to the rye telomeres probably due to the highly repetitive sequence present. In general, the probe specifically binding to the telomeres is not useful and should be avoided. However, in some circumstances it can be exploited to confirm that an introgression has both telomeres and is therefore almost certain to be a whole chromosome. Conversely, visualising the telomere proved to be useful when identifying chromosomes that had lost the most distal regions.

Potential future work

To further investigate these introgression using a cytogenetic approach. future work may take advantage of FISH. A FISH karyotype of the rye accessions used as germplasm donors could be produced using the introgression lines developed within this work. If rye specific sequences, such as pSc74, pSc250, and pSc200 (Fradkin *et al.* 2013), are used to produce the karyotype, it would be possible to simultaneously FISH both wheat and rye. This would enable the identification of which homoeologous group the rye introgression is from and which wheat chromosome the introgression has recombined/translocated with

5 Molecular marker investigation

5.1 Molecular marker Introduction

Molecular genotyping

Traditional introgression programs rely on cytogenetic techniques, which are limited by throughput and resolution, discussed above (chapter 4). Molecular techniques are increasingly being used as an effective alternative. The development of modern molecular techniques such as SNP arrays and next generation sequencing has enabled high through put and cost-effective genotyping in plant breeding programs (Thomson 2014). These technologies are being adapted for use in pre-breeding, and for the development of introgression lines (Tiwari *et al.* 2014; Wilkinson *et al.* 2016; Winfield *et al.* 2016; Grewal *et al.* 2017b; King *et al.* 2017). The effectiveness of using SNP markers to genotype wheat wild relatives introgressions was first demonstrated, using five flow sorted *Aegilops genicula* chromosomes by Tiwari *et al.* (2014).

Comparison of the Wheat and Rye genomes

To effectively map introgressions it is beneficial to understand the homoeologous relationship between the target crop, wheat, and the wild relative donor, rye. Within the grasses, species are closely related and gene order is often conserved (Gale and Devos 1998). Differences in gene order between the target plant and donor, such as translocations and inversions, can mean introgressions can be more difficult to identify, may cause an unbalanced genome and can limit recombination.

The wheat and rye genomes have been previously compared using RFLPs Devos *et al.* (1993a) and references therein. Chromosome one of rye was shown to be wholly collinear with wheat chromosome one and lacking translocations. All other rye chromosomes, two through seven, had changes in their chromosome structure and orthologous gene order complicating their homoeologous relationship with wheat. The work by Devos *et al.*

(1993a) has since been expanded upon using PLUG markers by Li *et al.* (2013) producing a more detailed comparative map.

Chapter aims

This chapter aims to genotype the Wheat-Rye introgression lines produced in chapter two using the Axiom® 35k wild relative SNP array [derived from the ultra-high-density Axiom® array (Winfield *et al.* 2016)] using the approach described in King *et al.* (2017) and in doing so produce a physical bin map of rye. A secondary aim is to compare the physical map with the wheat genome to investigate the homoeologous relationship of wheat and rye.

5.2 Axiom Materials and Methods

Plant material

Plants were obtained as described in chapter two along with two further wheat varieties Pavon 76 (idPlant: 20448, GRU Store Code: W7193) and Highbury (idPlant: 15111, GRU Store Code: W1576). A complete set of Imperial rye addition/substitution lines in a Chinese Spring background were obtained from Germplasm Resource Unit at the John Innes Centre (Chapter 2). Experimental introgression lines were produced as described in chapter three.

Bin Mapping

Whole genome DNA was extracted (chapter 2) and analysed with the 35 k Axiom® Wheat-Relative SNP Genotyping Array (Winfield *et al.* 2016; King *et al.* 2017). The array was developed to investigate 10 wheat wild relatives, (*Ae. caudata, Ae.speltoidies, Am. muticum, Th. bessarabicum, Th. elongatum, Th. intermedium, Th. ponticum, T. urartu, T.timopheevii* and *S. cereale)* and was derived from the Axiom® 820 K array (Wilkinson *et al.* 2016). The data set for the Axiom® 820 K array is available from http://www.cerealsdb.uk.net (Wilkinson *et al.* 2012; Wilkinson *et al.* 2016).

Two hundred and thirteen samples were analysed in total, including two *S. cereale* controls (accessions 390372 and 428373) and eight *T. aestivum* controls, i.e. two samples from each of the four cultivars used in the crossing programme (Chinese Spring, Paragon, Pavon 76 and Highbury). Samples were taken from the F_1, BC_1, BC_2 and BC_3 generations, grouped and analysed as a complete set to have a sufficient sample size. The data produced from the Axiom® Wheat-Relative array was analysed according to the protocol described in King *et al.* (2017) but using a call rate threshold of 80% instead of the a Axiom default of 97% in order to account for the lower rate of hybridisation of the wheat relatives to the array. The SNPs were clustered according to performance into six categories which were as follows: 'Poly High Resolution' (PHR), which were polymorphic and codominant with a minimum of two examples of the minor allele; 'No Minor Homozygote' (NMH) which were polymorphic and dominant, with two observed clusters; 'Mono High Resolution' (MHR) which were monomorphic; 'Off-Target Variant' (OTV) with four clusters and one representing a null allele; 'Call Rate Below Threshold' (CRBT) the SNP call rate was below threshold but other cluster properties were above threshold; and 'Other' where one or more cluster properties were below threshold, figure 5.1 shows the clustering of each calling categories and was produced by Hussain *et al.* (2017) The PHR SNPs were used for genotyping, physical mapping and comparing gene orthologous gene order as they have good cluster resolution and each SNP behaves as a diploid.

Unsuitable markers were removed from the PHR SNPs using Flapjack™ software (Milne *et al.* 2010), leaving only markers that indicated the presence of rye chromatin. Markers were removed for the following reasons: (i) the calls were polymorphic between the wheat controls; (ii) the calls were polymorphic between the rye controls; (iii) there was no call for either the wheat and/or rye controls; (iv) there was no polymorphism between the wheat and rye controls.

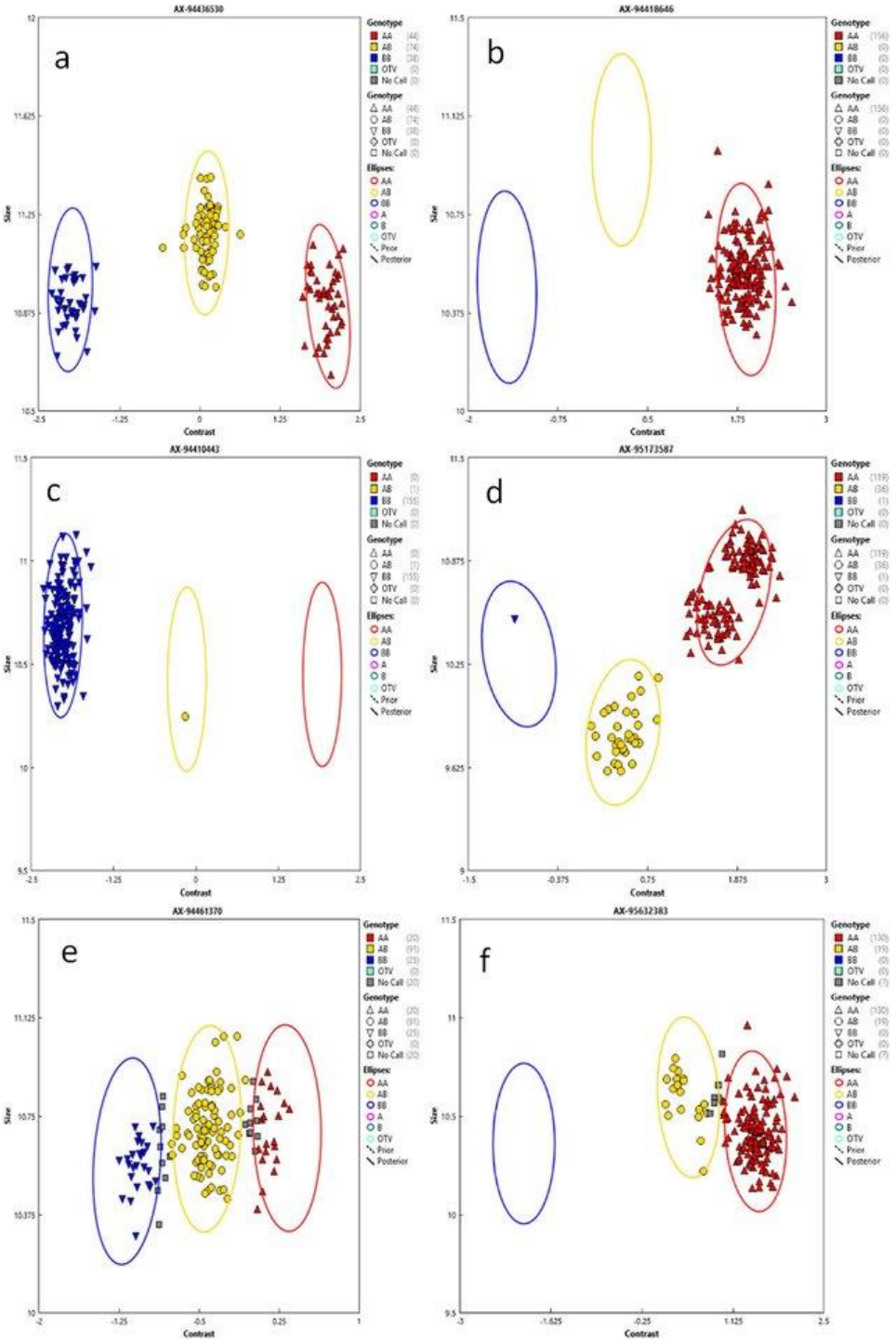


Figure 5.1 Representations of the six SNPs calling categories: (a) Poly High Resolution; (b) Mono High Resolution; (c) No Minor Homozygote; (d) Off-Target Variants (e) Call Rate Below Threshold; and (f) Other
from (Hussain *et al*. 2017)

The remaining markers were grouped in JoinMap® 4.1 based on the recombination pattern of the markers(van Ooijen 2011) using a LOD score of 30 and a recombination frequency threshold of 0.05 with the Haldane mapping function (Haldane 1919), meaning only highly similar groups of markers with similar recombination patterns were grouped, a high LOD score was used because of the variability between the wheat and the rye genomes. The highest ranked linkage groups with more than 25 markers were used for map construction. Markers that were unlinked and/or did not show a heterozygous call were ignored.

The sequence of each marker was compared to the wheat genome (http://plants.ensembl.org/Triticum_aestivum) using BLAST with a cut off e-value of 1e-05. The linkage group and chromosome arm of each marker was identified according to the IWGSC wheat survey sequence v3 (The International Wheat Genome Sequencing Consortium 2014). Blast data and information from the Axiom® Wheat HD Genotyping Array (Winfield *et al.* 2016) was used to assign markers to chromosome arms and combine the original Joinmap groups into 7 linkage groups representative of the seven rye chromosomes with respect to the known structural differences between wheat and rye (Devos *et al.* 1993a; Ishikawa *et al.* 2007). The seven linkage groups were validated with Axiom® array data from the Imperial rye addition lines.

Markers within the seven linkage groups were initially ordered with JoinMap 4.1 (van Ooijen 2011) and then manually reordered based on physical map positions from blast and placed in physical bins based on size of the rye introgressions and chromosomal aberrations. Aberrations included any abnormal chromosomes and were mostly fragments of rye chromosomes. The physical bin map was confirmed using the single colour GISH data produced in chapter 4 i.e., by comparing the number and size of rye introgressions visualised in GISH images with the genotypes of the same line. Within each bin the markers were ordered according to their position

within the wheat genome according to the IWGSC Chinese Spring genome sequence Refseq v1 (IWGSC 2018).

Visualisation of genotypes using GGT

Genotype data was imported into Microsoft excel and formatted for GGT 2.0 (van Berloo 2008) according to the software's parameters. Since genetic distance data was not available for these markers, the bin position of markers was used to input in GGT and assigned in single digit increments with the first marker assigned as one and the second as two and so on. Markers within 10,000 bp of each other on the wheat pseudomolecules were assigned the same bin position number. The actual physical position of these markers was not used because the physical position data was gathered from wheat and does not reflect the structural differences and translocations between wheat and rye. The genotype data was prepared in Microsoft excel was imported into GGT2.0 and images were exported as Jpegs.

Comparative (syntenic) Analysis

The marker sequence order was compared between the bin map of rye described here and the wheat genome. Marker sequences were compared to the wheat genome reference sequence Refseq v1 using BLAST (as described above) with the orthologous position of the top hit of each of the wheat A, B and D genomes being taken. To simplify comparison, the A and B genome were ignored as the D genome had the largest number of overall top hits. The markers within the bin map were given a value starting from one and increasing in increments of one per marker. Markers with IWGSC positions with less than 10,000bp difference were given the same value. The binned marker values were then scaled up by a factor of 1,000,000 to be comparable to the physical positions of the wheat D genome in Mb. The comparison between the two genomes was visualised using Circos (Krzywinski *et al.* 2009).

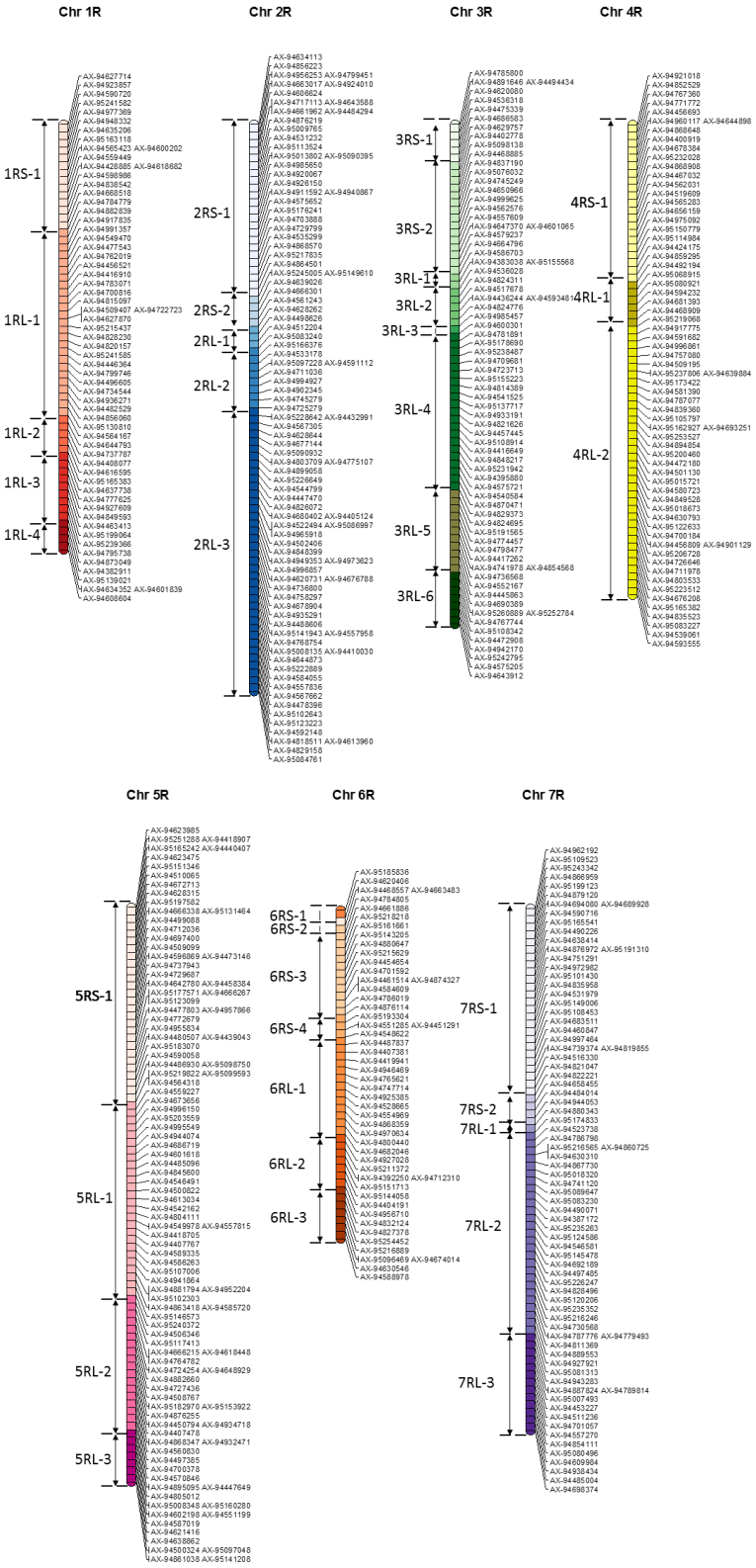


Figure 5.2. A bin map of rye consisting of 545 markers in 37 bins. Bins are labelled to the left of each chromosome linkage group and marker names are shown the right. Bin names ending in S indicate bins on the short arm of the chromosome and names ending in L indicate bins on the long arm of the chromosomes.

5.3 Axiom Results

Bin mapping

A total of 2,609 PHR markers were obtained for rye; 535 of these PHR markers were assigned to a rye bin map. Therefore, 7.2% of the SNP markers on the 35K axiom array were useable as rye PHR markers. 20.9% of PHR markers were successfully assigned to the genetic bin map. Thus, from the 35 K axiom 1.5% of the total markers were used to create the genetic bin map.

Seven linkage groups were produced representing each rye chromosome. Markers were separated into 37 bins across all linkage groups. Bin start and end points were determined using introgressions, including recombinants, aberrations, and telocentric chromosomes. The available Imperial rye addition lines were used as positive controls for as many of the linkage groups as possible. Linkage groups were named 1R for linkage group 1, 2R for linkage group 2, 3R for linkage group 3, etc. A breakdown of markers and bins per linkage group is shown in table. 5.1. The whole bin map was visualised with MapChart 2.3 (Voorrips 2002)(Figure 5.2).

Chromosome 3R had the highest number of bins with 8 bins and chromosome 4R the lowest with only 3 bins. The average number of bins per chromosome was 5.

Table 5.1 The number of markers and bins in each linkage group.

	1R	2R	3R	4R	5R	6R	7R
Markers	63	96	74	68	105	51	78
Bins	5	5	8	3	4	7	5

Comparison of the wheat and rye genome (Synteny)

The bin map was compared to the wheat D-genome, by comparing bin map marker position with its sequence position within the wheat D-genome. The comparison was visualised with Circos (Krzywinski *et al.* 2009) as shown in figure 5.3. The comparison shows a lack of coverage around the centromere in all seven linkage groups. Figure 5.4 Is a chromosome map of the seven rye lnkage groups with the orthologous regions from wheat shown.

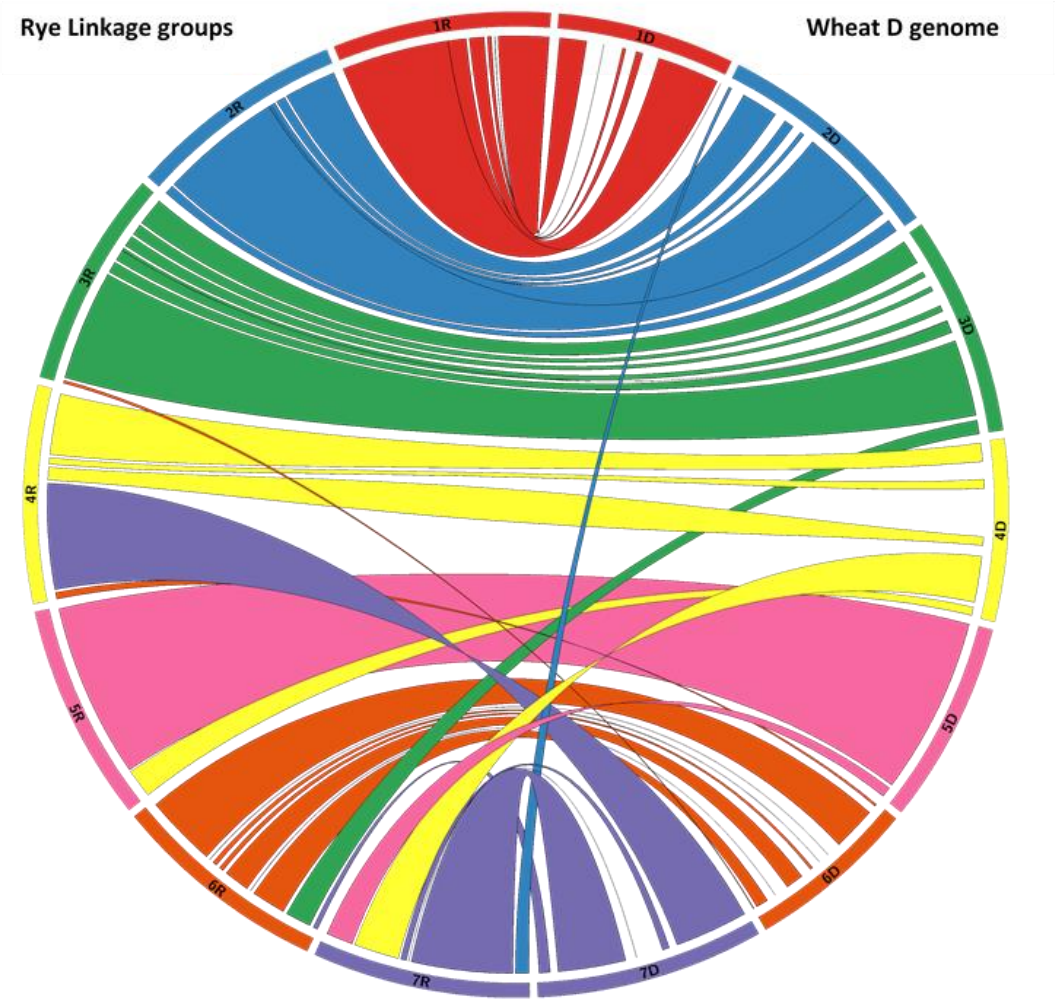


Figure 5.3 Comparative positions of marker sequences in rye (positions according to the bin map shown in Figure5.1) and the D genome of hexaploid wheat (physical positions in Mb); visualized with Circos v. 0.67 (Krzywinski *et al.* 2009).

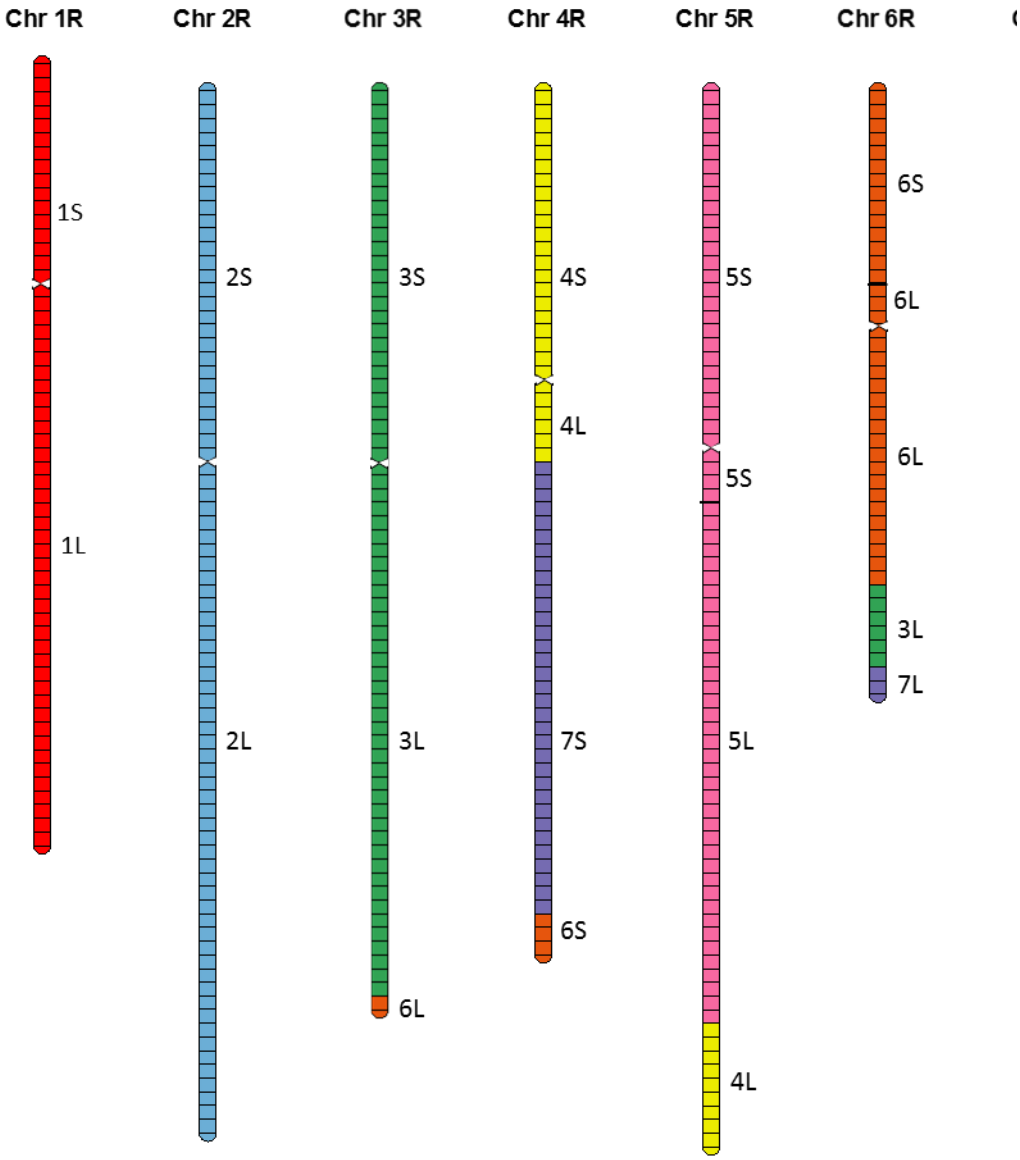


Figure 5.4 A representation of the *S. cereale* chromosomes showing their homoeologous relationship with wheat. Chromosomes were constructed according to the bin map (Figure 5.2) and coloured according to the wheat chromosome each marker sequence is mapped to.

Linkage groups 1R and 2R

Linkage groups 1R and 2R show a conserved marker sequence order with wheat with no evidence of translocations from other wheat chromosomes.

Linkage group 3R

Linkage group 3R has a largely conserved marker sequence order with wheat chromosome 3D, with a small translocation from wheat chromosome 6 translocated to the distal end of the short arm (Figure 5.5)

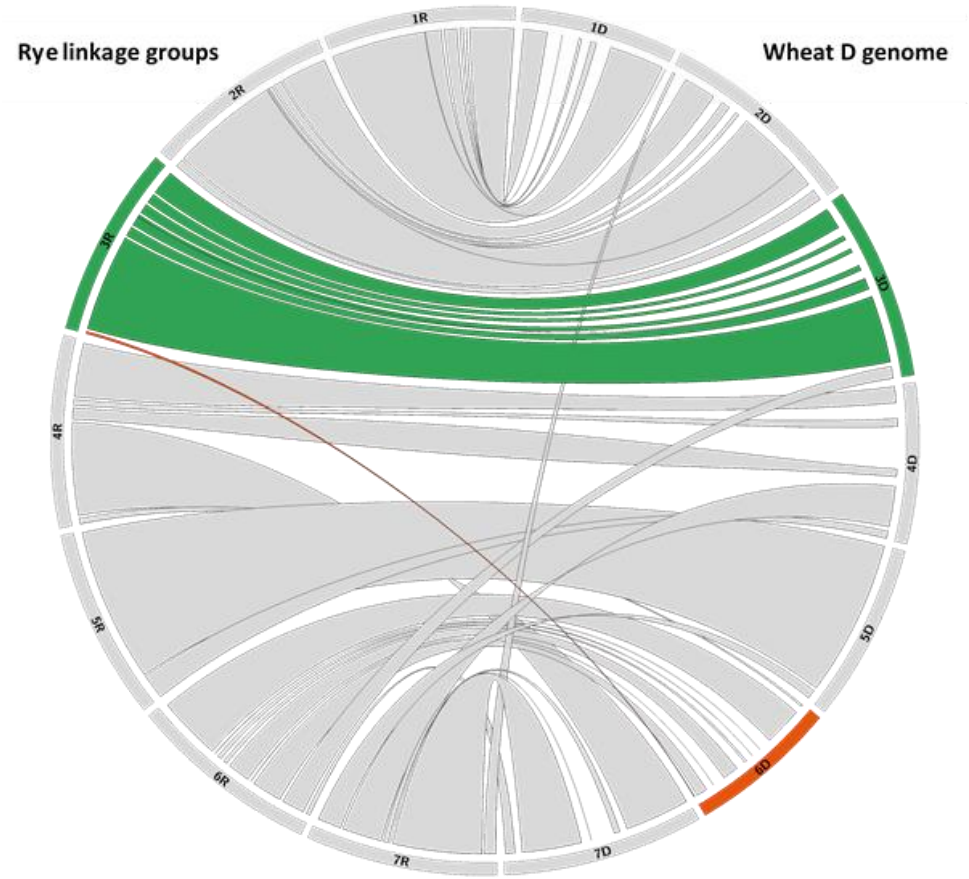


Figure 5.5 Comparative positions of marker sequences in rye linkage group 3R (positions according to the bin map – Figure 5.2) with the D genome of hexaploid wheat (physical positions in Mb); visualized with Circos v. 0.67 (Krzywinski *et al.* 2009). Markers related to rye linkage group 3R are coloured.

Linkage group 4R

Linkage group 4R shows considerable change in comparison to wheat chromosome 4. The short arm of linkage group 4R maps to the short arm of wheat chromosome 4. The long arm of linkage group 4R maps to the proximal region of wheat 4L, a large interstitial group of wheat 7S and a distal group of wheat 6S (Figure 5.6).

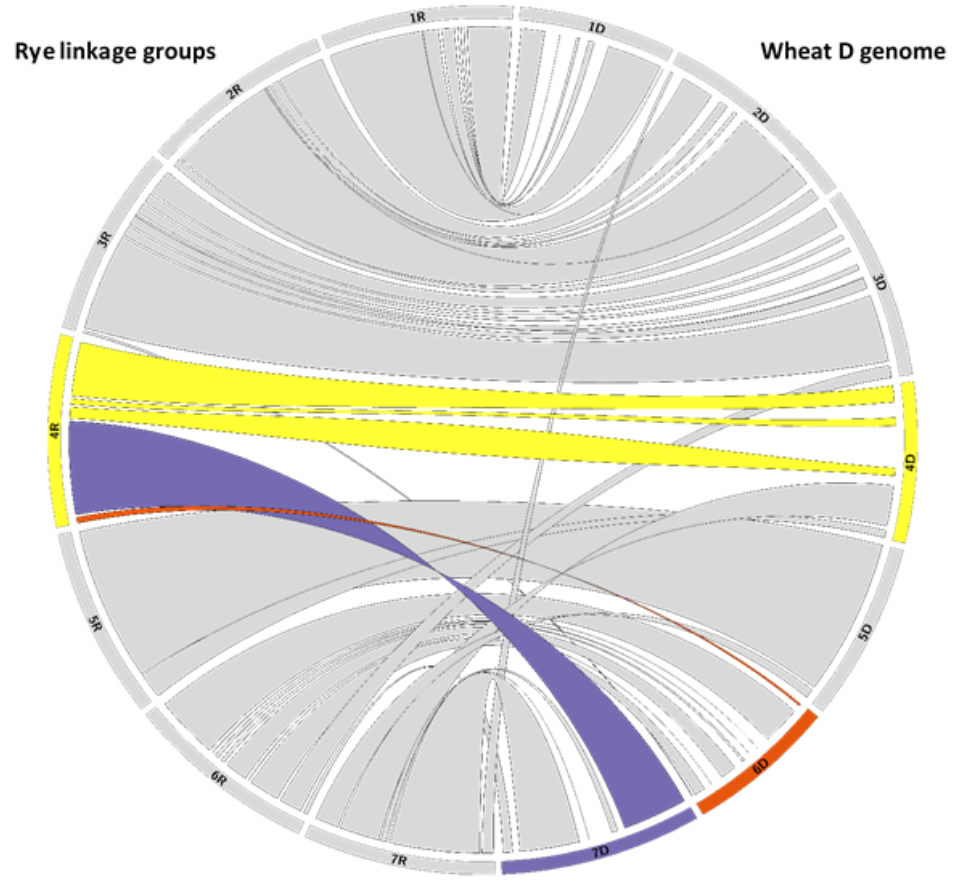


Figure 5.6 Comparative positions of marker sequences in rye linkage group 4 (positions according to the bin map -Figure 5.2) with the D genome of hexaploid wheat (physical positions in Mb); visualized with Circos v. 0.67 (Krzywinski *et al.* 2009). Markers related to rye linkage group 4 are coloured.

Linkage group 5R

Most of linkage group 5R shares its orthologous markers with wheat chromosome 5D. A group from wheat chromosome arm 4L maps to the distal end of the long arm of linkage group 5R (Figure 5.7).

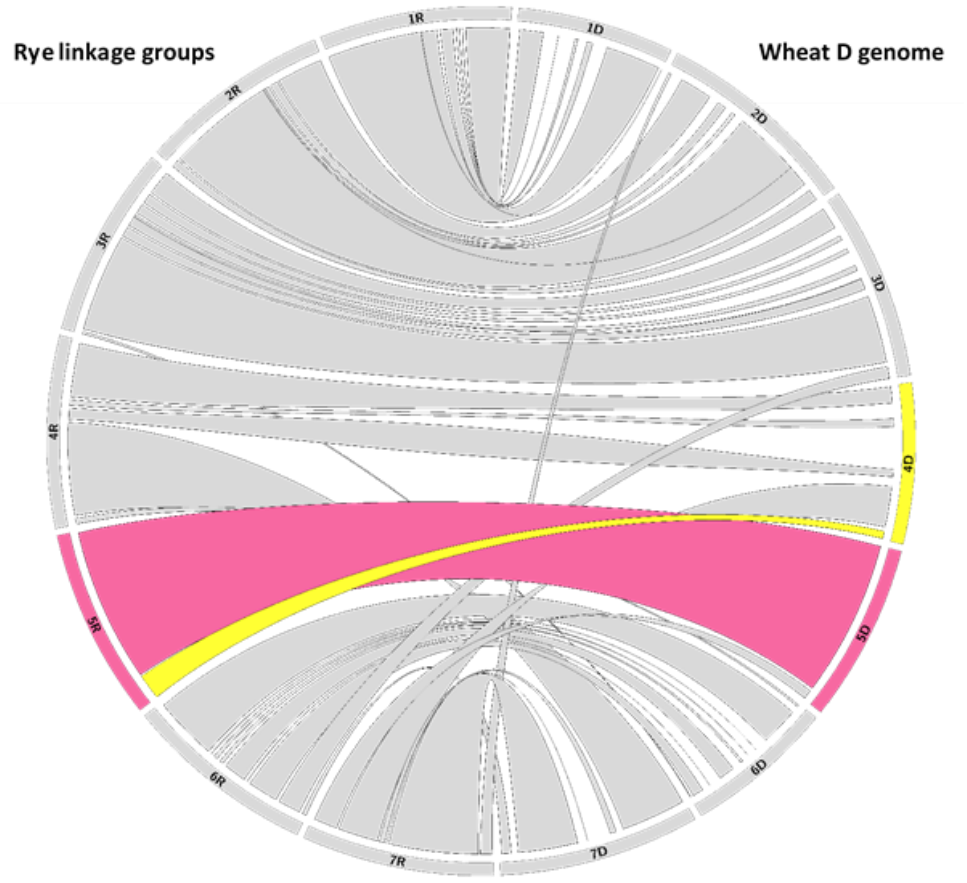


Figure 5.7 Comparative positions of marker sequences in rye linkage group 5 (positions according to the bin map – Figure 5.2) with the D genome of hexaploid wheat (physical positions in Mb); visualized with Circos v. 0.67 (Krzywinski *et al.* 2009). Markers related to rye linkage group 5 are coloured.

Linkage group 6R

The short arm of linkage group 6R maps mostly to wheat chromosome 6S and a small proximal region to chromosome 6L. The long arm of chromosome 6R maps to wheat chromosome arms 6L, 3L and 7L, order from most proximal to most distal (Figure 5.8).

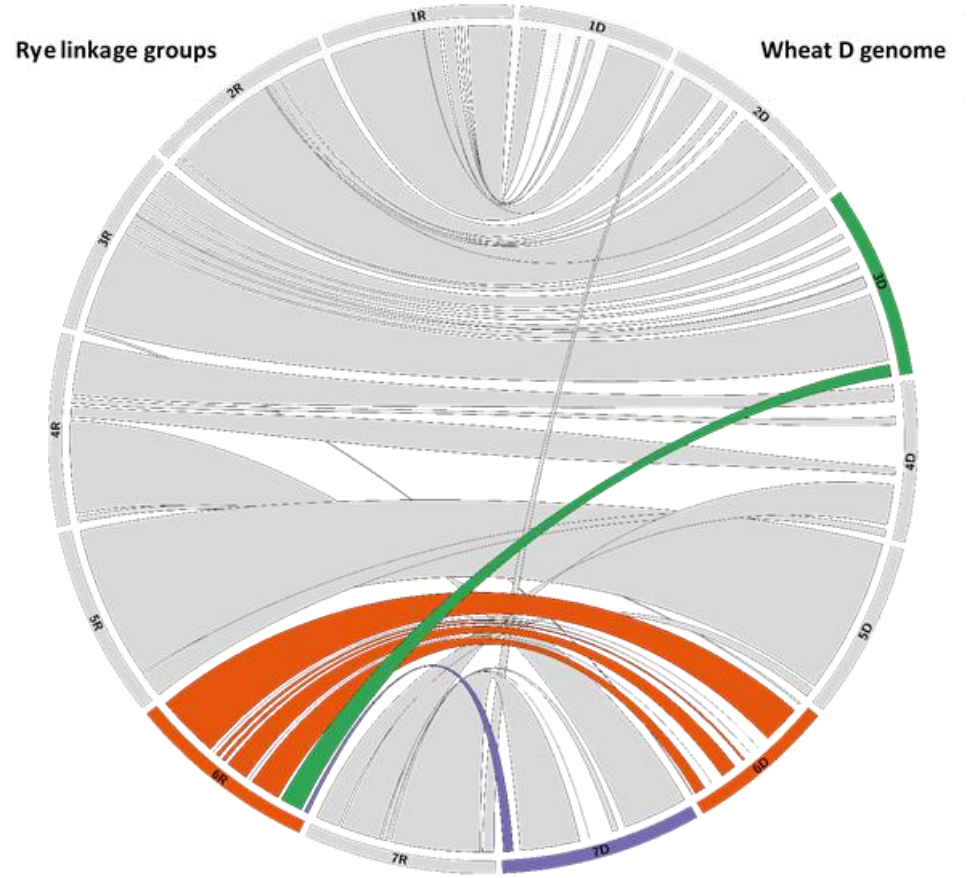


Figure 5.8 Comparative positions of marker sequences in rye linkage group 6 (positions according to the bin map – Figure 5.2) with the D genome of hexaploid wheat (physical positions in Mb); visualized with Circos v. 0.67 (Krzywinski *et al.* 2009). Markers related to rye linkage group 6 are coloured.

Linkage group 7R

The short arm of linkage group 7R maps to a distal region of wheat 5L, an interstitial region of wheat 4L and a proximal region of wheat 7S. The long arm of linkage group 7R maps to wheat 7L with a distal region of wheat 2S (Figure 5.9).

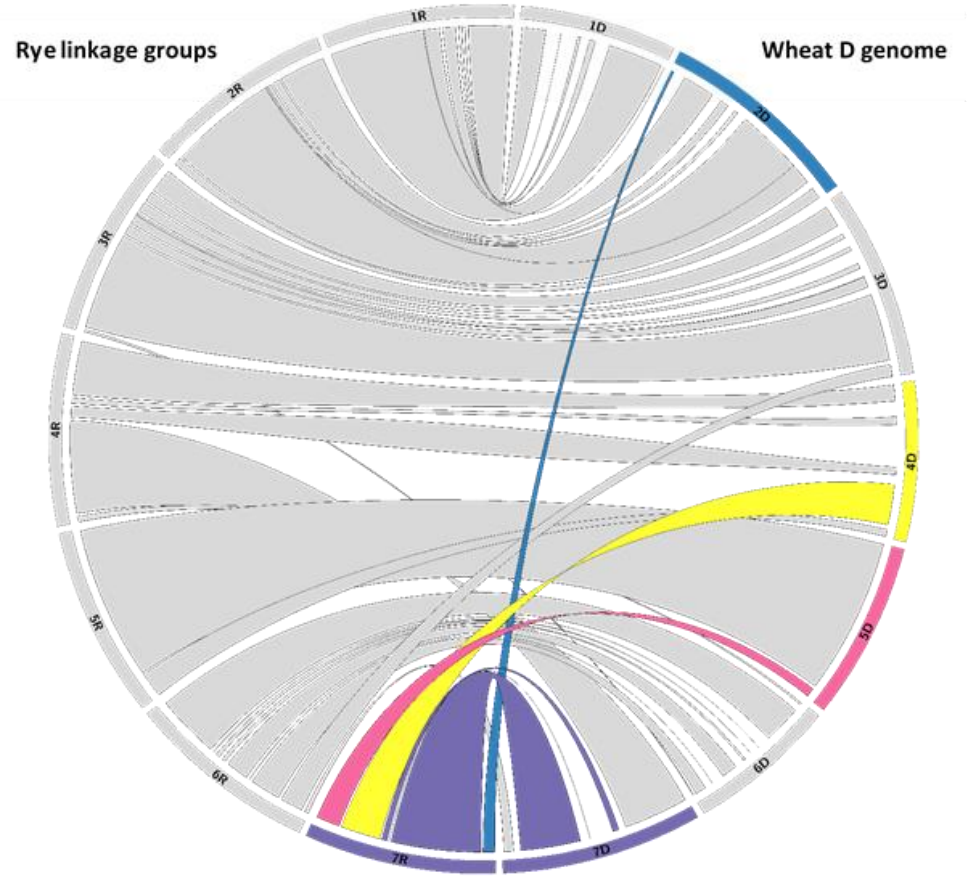


Figure 5.9 Comparative positions of marker sequences in rye linkage group 7 (positions according to the bin map – Figure 5.2) with the D genome of hexaploid wheat (physical positions in Mb); visualized with Circos v. 0.67 (Krzywinski *et al.* 2009). Markers related to rye linkage group 7 are coloured.

Identification of introgressions

A total of 188 introgression lines were genotyped. Of these 188, three were F_1 lines. In the genotyped F_1s every marker in all seven linkage groups indicated the presence of rye chromatin. The F_1s were excluded from further analysis and therefore 185 lines were used for further analysis.

A total of 535 introgressions were identified using the Axiom® 35k Wild-Relative Genotyping array. Table 5.2 shows the number of introgressions per linkage group and subdivides the introgressions into four categories; [whole chromosomes (all markers in a linkage group indicate rye), long arm (all the markers on the long arm indicate rye but no further markers), short arm (all short arm markers indicate rye but no further markers) and other (any introgression that does not fit into the above categories. The 'other' introgressions are described in more detail below.

Table 5.2 The number of introgressions identified using the Axiom 35 k wild relative array separated according to linkage group. The number of experimental lines containing introgressions is shown with the percentage of experimental lines containing an introgression. The introgressions are then subdivided according to type, (Whole chromosomes, complete long arm only, complete short arm only, and other) the number of introgressions is given as well as the percentage of the total introgressions.

	1R	2R	3R	4R	5R	6R	7R
Total Introgressions	83	76	79	69	68	81	79
(% of all lines)	(44.8)	(41.1)	(42.7)	(37.3)	(36.8)	(43.8)	(42.7)
Whole Chromosome	72	67	67	60	61	61	73
(% of introgressions)	(85.5)	(88.2)	(84.8)	87.0	(89.7)	(75.3)	(92.4)
Long arm	6	5	2	7	3	1	1
(% of introgressions)	(7.2)	(6.5)	(2.5)	(10.1)	(4.4)	(1.2)	(1.3)
Short arm	1	0	4	1	1	0	2
(% of introgressions)	(1.2)	(0.0)	(5.1)	(1.4)	(1.5)	(0.0)	(2.5)
Other	5	4	6	1	3	19	3
(% of introgressions)	(6.0)	(5.2)	(7.5)	(1.4)	(4.4)	(23.5)	(3.8)

The relative sizes of introgressions stated below is based on the bin mapping of markers re-ordered in respect to the translocations found in rye and therefore it is not representative of actual physical distances and the genetic distances are likely to be highly skewed.

Out of the 85 1R introgressions, five were not full or half chromosomes. Three of the five were from the same family and with the same introgression named 1Ri. 1Ri contains the whole of 1RS and all but the distal region of 1RL; 1Ri covered 60 markers and 95.2% of 1R (it is important to note this is the genetic distances and the actual physical distance will differ). The two remaining introgressions, 1Rii and 1Riii were distinct. 1Rii contained the whole of 1RS and proximal and distal regions of 1RL, covered 53 markers and 85.7% of 1R. 1Riii contained the whole of 1RS and the proximal region of 1RL, covered 44 markers and 69.8% of 1R.

Out of the 76 2R introgressions, four were not full or half chromosomes. All four were distinct introgressions named 2Ri, 2Rii, 2Riii and 2Riv. 2Ri contained the whole of 2RL and the two most proximal markers of 2RS, covered a total of 62 markers and 64.6% of 2R. 2Rii contained the whole of 2RS and a 12 marker region of proximal 2RL, covered a total of 48 markers and 50% of 2R. 2Riii contained the whole of 2RS and a marker region of proximal 2RL, covering a total of 39 markers and 40.6% of 2R. 2Riv covers the majority of 2RS except a region of 4 proximal markers, covering a total of 32 markers and 33.3% of 2R.

Out of the 79 3R introgressions, six were not full or half chromosomes. The six were then sorted into five groups; 3Ri, 3Rii, 3Riii, 3Riv and 3Rv. All groups contained one introgression line except 3Riii that contained two lines from the same family. 3Ri contained the whole of 3RS and a three marker region of proximal 3RL, covered a total of 26 markers and 35.1% of 3R. 3Rii contained the whole of 3RS and an eight marker region of proximal 3RL, covered a total of 31 markers and 41.9% of 3R. 3Riii contained the whole of

3RS and a nine marker region of proximal 3RL, covered a total of 32 markers and 43.2% of 3R. 3Riv contained the whole of 3RS and a 44 marker region of 3RL only missing the seven most distal markers and therefore covered a total of 67 markers and 90.5% of 3R. 3Rv contained the whole of 3RL and all but the most distal seven markers of 3RS, covered 67 makers and 90.5% of 3R.

Out of the 69 4R introgressions, only one was not a full or half chromosome. This introgression was named 4Ri. 4Ri covered the whole of 4RS and a six-marker region of proximal 4RL, covered a total of 29 markers and 42.0% of 4R.

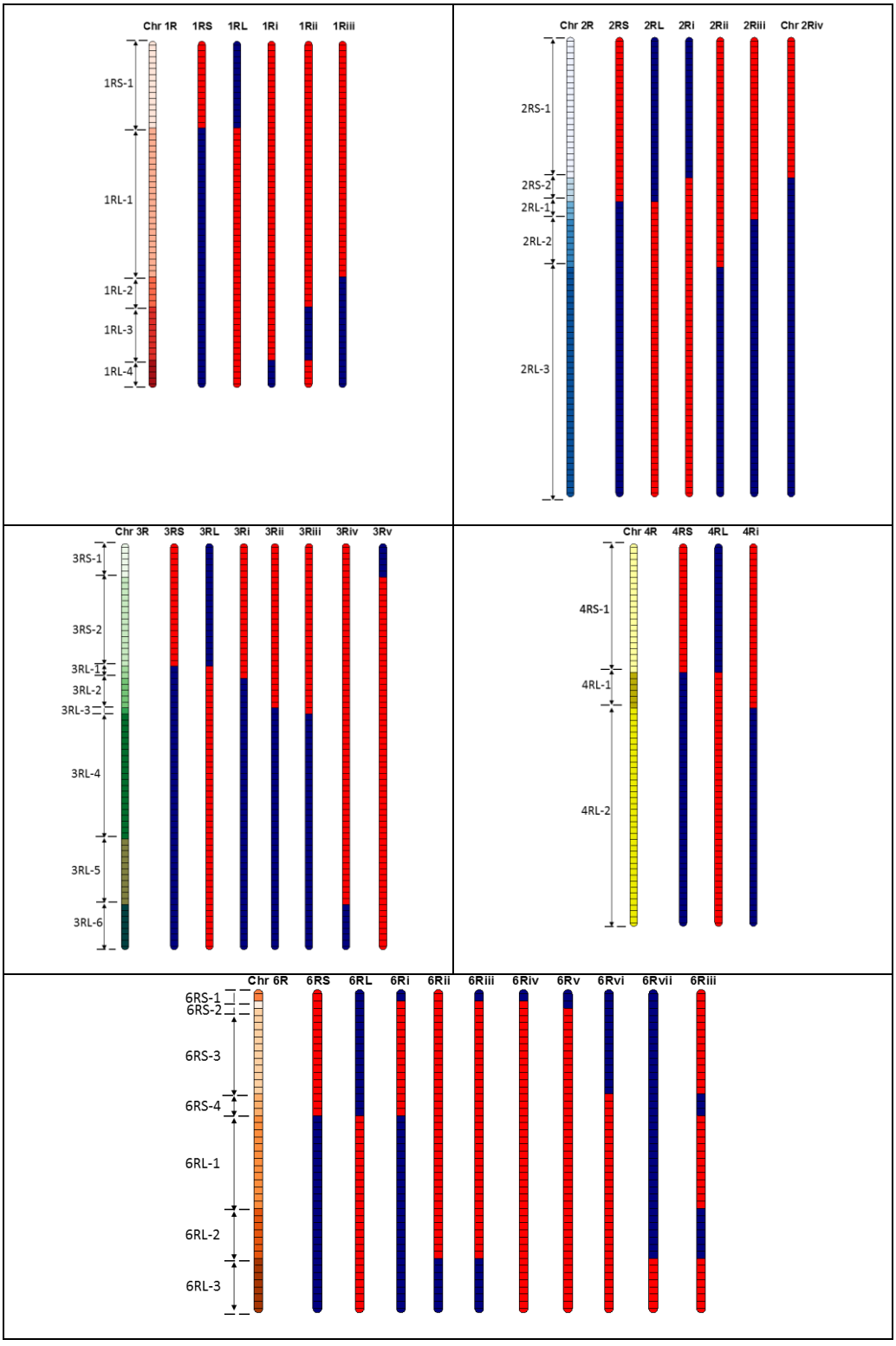
Out of the 68 5R introgressions, three were not full or half chromosomes. The three introgressions were sorted into two groups; 5Ri and 5Rii. 5Ri consisted of two lines from the same family with the same sized introgression. The 5Ri introgression covered all 5R except the distal end on 5RL, covered a total of 94 markers and 89.5% of 5R. 5Rii was found in a single line and contained the whole of 5RS and a large proximal region of 5RL, covered a total of 69 markers and 65.7% of 5R.

Out of the 81 6R introgressions, 19 were not full chromosomes and did not exactly match the chromosome arms. The 19 introgressions were then sorted into eight groups; 6Ri (single line), 6Rii (eight lines), 6Riii (five lines), 6Riv (single line), 6Rv (single line), 6Rvi (single line), 6Rvii (single line) and 6Rviii (single line). Group 6Ri was composed of all but the two most distal markers of 6RS. The 6Rii introgression contained the whole of 6RS and all but eight distal markers of 6RL and covered a total of 43 markers and 84.3% of the chromosome 6R. 6Riii was composed of the whole of 6RS except two distal markers and all but eight distal markers of 6RL and covered a total of 41 markers and 80.4% of the chromosome 6R. The 6Riv introgression was composed of the whole of 6R except the two most distal markers from 6RS and covered 49 markers and 96.1% of 6R. 6Rv covered the whole of 6R

except the four most distal markers in 6RS, covered 47 markers and 92.2% of 6R. 6Rvi was made up of the whole of 6RL and the four most proximal markers in 6RS, covered a total 33 markers and 64.7% of 6R. The 6Rvii introgression, was composed of 16 markers from the distal region of 6RL and covered 31.4% of 6R. 6Rviii covered the whole of 6R except for two medial groups of four and three markers respectively and covered 44 markers and 86.3% of 6R.

Of the 79 7R introgressions, three were not full chromosomes or chromosome arms. All three introgressions were individuals and named 7Ri, 7Rii and 7Riii. 7Ri covered the whole of 7RS and the first proximal marker of 7RL and covered a total of 34 markers and 43.6% of 7R. 7Rii covered the whole of 7RS except four proximal markers and covered a total of 29 markers and 37.2% of 7R. 7Riii was a distal introgression of 7RL and covered 14 markers and 17.9% of 7R.

Figure 5.10 shows how the wheat rye introgression lines described above aligned to the physical bin map (figure 5.2) and shows the relationship between the bin size and the introgressions produced (the bins are delimited by the size of the introgressions).


Chr 1R
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1Ri
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1Riii
1RS-1
1RL-1
1RL-2
1RL-3
1RL-4
Chr 2R
2RS
2RL
2Ri
2Rii
2Riii
Chr 2Riv
2RS-1
2RS-2
2RL-1
2RL-2
2RL-3
Chr 3R
3RS
3RL
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3RS-1
3RS-2
3RL-1
3RL-2
3RL-3
3RL-4
3RL-5
3RL-6
Chr 4R
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4RS-1
4RL-1
4RL-2
Chr 6R
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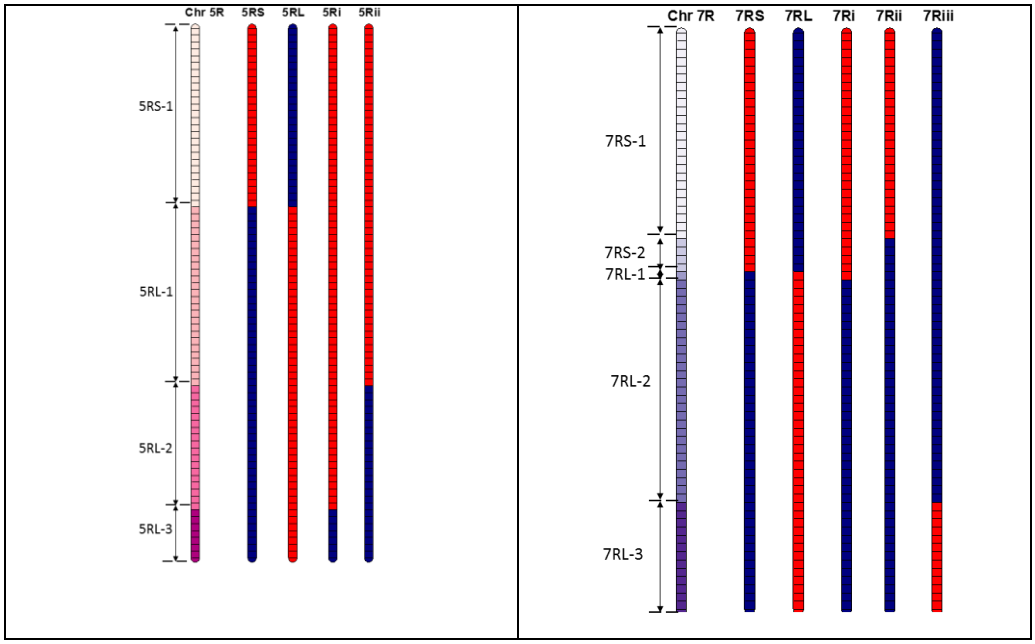


Figure 5.10 The rye physical bin map (produced as described above) aligned against the wheat rye introgressions, dected using the affymetix wild relative SNP array. Chromosome from the bin map have been seprated. The first image in each box shows the Rye physical bin map for each chromosome, coloured accoriding to the bin map (figure 5.2). The remaining images are coloured accoring to the presence of rye introgressions, with blue regions representing wheat alleles and red regions representing rye alleles.

5.4 Axiom Discussion

Physical Bin Map

The physical bin map presented covers all 7 rye chromosomes. The comparative analysis suggests a lack of coverage around the centromere of six of the chromosomes with only group 5 showing a better coverage of the centromere.

The physical bin map produced via the Axiom array genotyping enabled the rapid identification of rye chromatin present within a wheat background and the ability to track introgressions through successive generations (Figure 5.11).

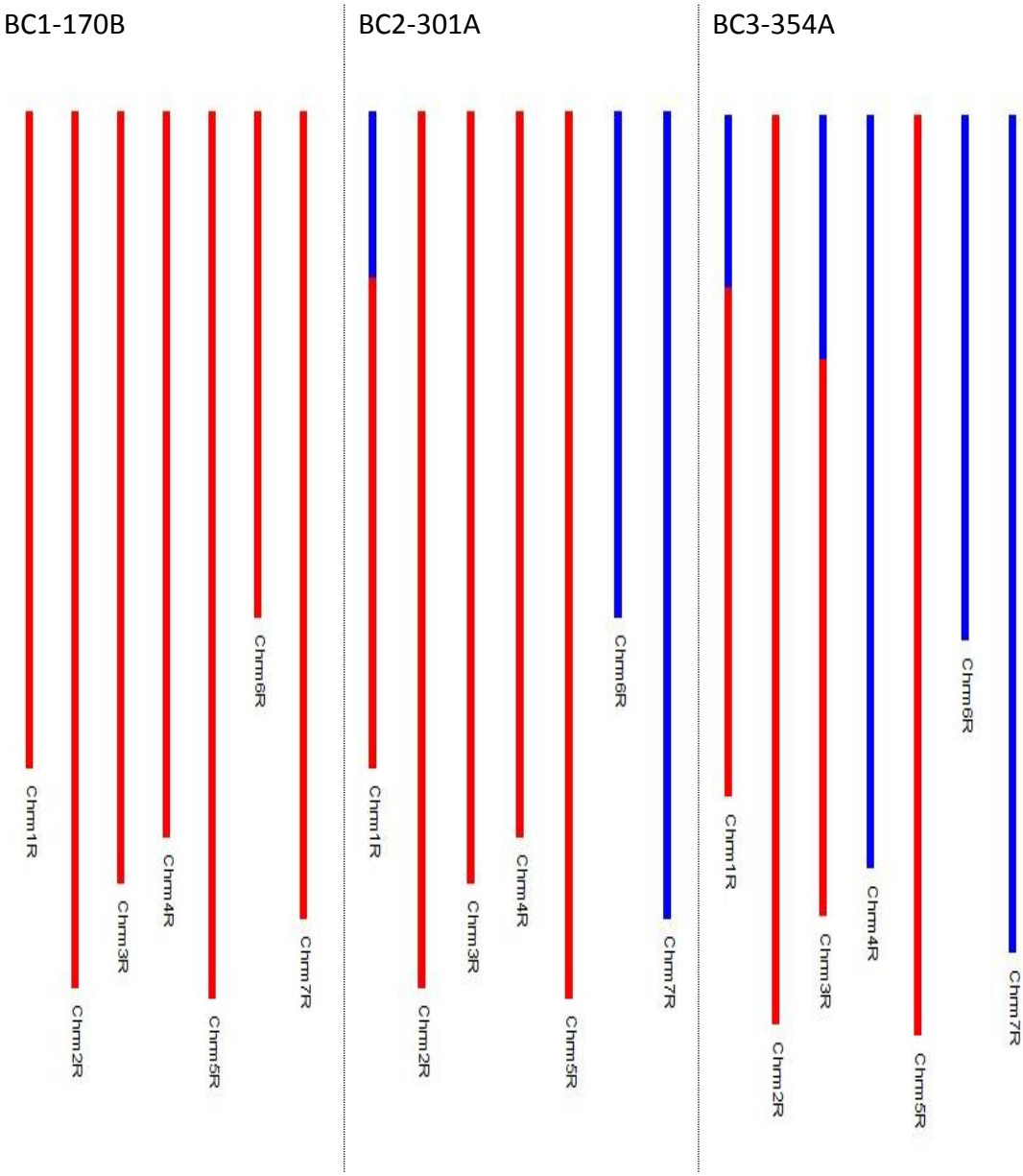
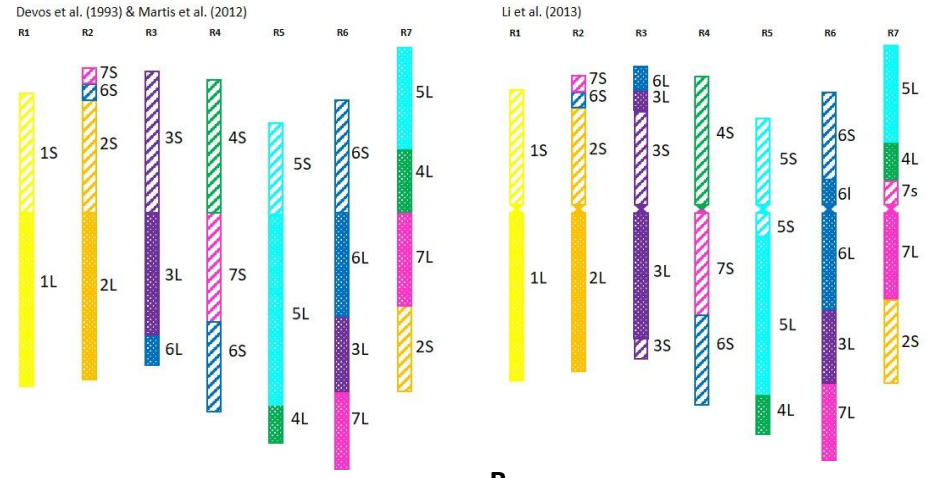


Figure 5.11 Images made in the GGT 2.0 (van Berloo 2008) software showing a family of introgression lines. Red bars indicate the regions of rye chromatin, blue bars indicate the regions with only wheat chromatin. GGT 2.0 visualises the genotype of a line

The genotyping was also tested on a closely related species to *S. cereale*. Two *Secale iranacum* introgression lines (developed as in chapter 2), were tested using the rye *(Secale cereale)* map. Results showed that the genetic map could not be used for *S. iranacum* due to differences in the *S. iranacum* and *S. cereale* genomes. However, the Secale species are diverse and therefore it would be worthwhile to test the genotyping on a range of Secale species.

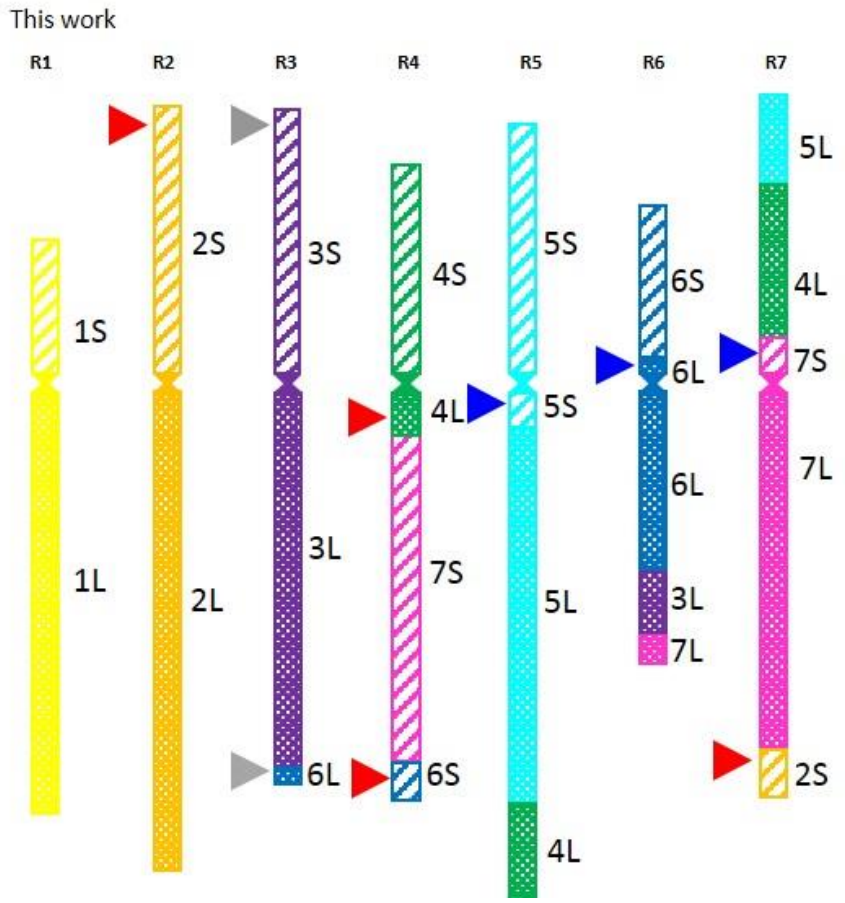
Comparative mapping

Devos *et al.* (1993a) compared the homoeologous relationships of wheat and rye using RFLP mapping which was then expanded upon by Martis et al. (2013) using a SNP array, Li *et al.* (2013) compared the same relationship using a modified version of EST markers (PLUG). Figure 5.11 shows the maps produced by Devos *et al.* (1993)/Martis *et al.* (2013) and Li *et al.* (2013) and compares them to the map produced in this work. In general, the same homoeologous relationships as previously reported were found. The Martis *et al.* (2013) map has been further confirmed by the recent mapping of the Rye genomes by Bauer et al. (2017), differences are described below.



A

B



C

Figure 5.12 Comparative maps showing the homoeologous relationship between wheat and rye. Map A was produced by Devos *et al*. (1993a) and confirmed by Martis *et al*. (2013). Map B was produced by Li *et al*. (2013). Map C was produced in the work presented here. Arrows indicate differences between maps. Red arrows are changes in map C when compared to both A and B. Blue are changes from A. Grey are changes from B. It should be noted in map A, on the short arm of linkage group 7R, there is a small region of 7S at the centromere that was too small to visualize.

1R

Chromosome 1R showed only markers mapping to homoeologous group 1 of wheat, agreeing with all previous studies.

2R

Chromosome 2R showed only markers mapping to wheat homoeologous group 2. This disagrees with the results from Devos *et al.* (1993a), Martis *et al.* (2013), Bauer *et al.* (2017) and Li *et al.* (2013). Devos *et al.* (1993a) and Li *et al.* (2013) both showed a 7S and 6S terminal translocation onto 2S. Were as Bauer et al. (2017) and Martis *et al.* (2013) show a 7S terminal translocation. It is possible this translocation was not present within the rye accessions used in this breeding programme. However, this is unlikely, as the addition lines used as controls were Imperial rye in a Chinese Spring background and these were also used in Devos et al. (1993a). However, it should be remembered that rye is an outbreeder and therefore can be polymorphic (Alkhimova et al. 1999). The most likely explanation for the lack of the 7S and 6S translocation is because they are both relatively small terminal translocations and thus were not detected by the markers in this study, i.e. marker coverage was not total.

3R

Chromosome 3R mapping of introgression material showed the same homoeologous relationship as reported in Devos *et al.* (1993), Martis *et al*. (2013) and Bauer *et al.* (2017) but lacks the secondary translocation of 3L and 6L onto 3S reported in Li *et al.* (2013). Interestingly the mapping of the 3RS telocentric Imperial rye addition line did show this translocation and therefore agreed with the mapping in Li et al (2013). This suggests that this translocation may be accession dependent and therefore likely to be a recent translocation. Thus, the mapping of the Imperial rye addition lines here differs to the mapping of Imperial rye undertaken in Devos *et al.* (1993)

and may be explained by cytogenetic polymorphism in the Imperial rye addition lines (Alkhimova *et al.* 1999).

4R

The mapping of chromosome 4R is mainly in agreement with the previous work although this study does show two differences to what has been previously reported. Firstly, the terminal region of 6S seems to be a smaller region than previously reported. This may again be due to a lack of terminal markers in 4RL. Secondly, a region of 4L was shown to be present proximal to the centromere in 4RL, confirming the hypothesis given by Devos *et al.* (1993) that a small region of 4L was present in 4RL and the 4/7 translocation is not centromeric.

5R

The mapping of chromosome 5R agrees with the mapping in Li *et al.* (2013), including the presence of a region of 5S proximal to the centromere in 5RL that was not reported in Devos *et al.* (1993). Bauer *et al.* reported a second region of reduced recombination frequency (usually found at the centromere) in 5RL and theorised the cause to be neocentric activity (a second region acting as a centromere) previously described in (Schlegel 1987; Manzanero et al. 2000), this is supported by the findings described here showing a region of 5RS present in 5RL that is likely to region of the neocentric activity caused by a disruption of the centromere.

6R

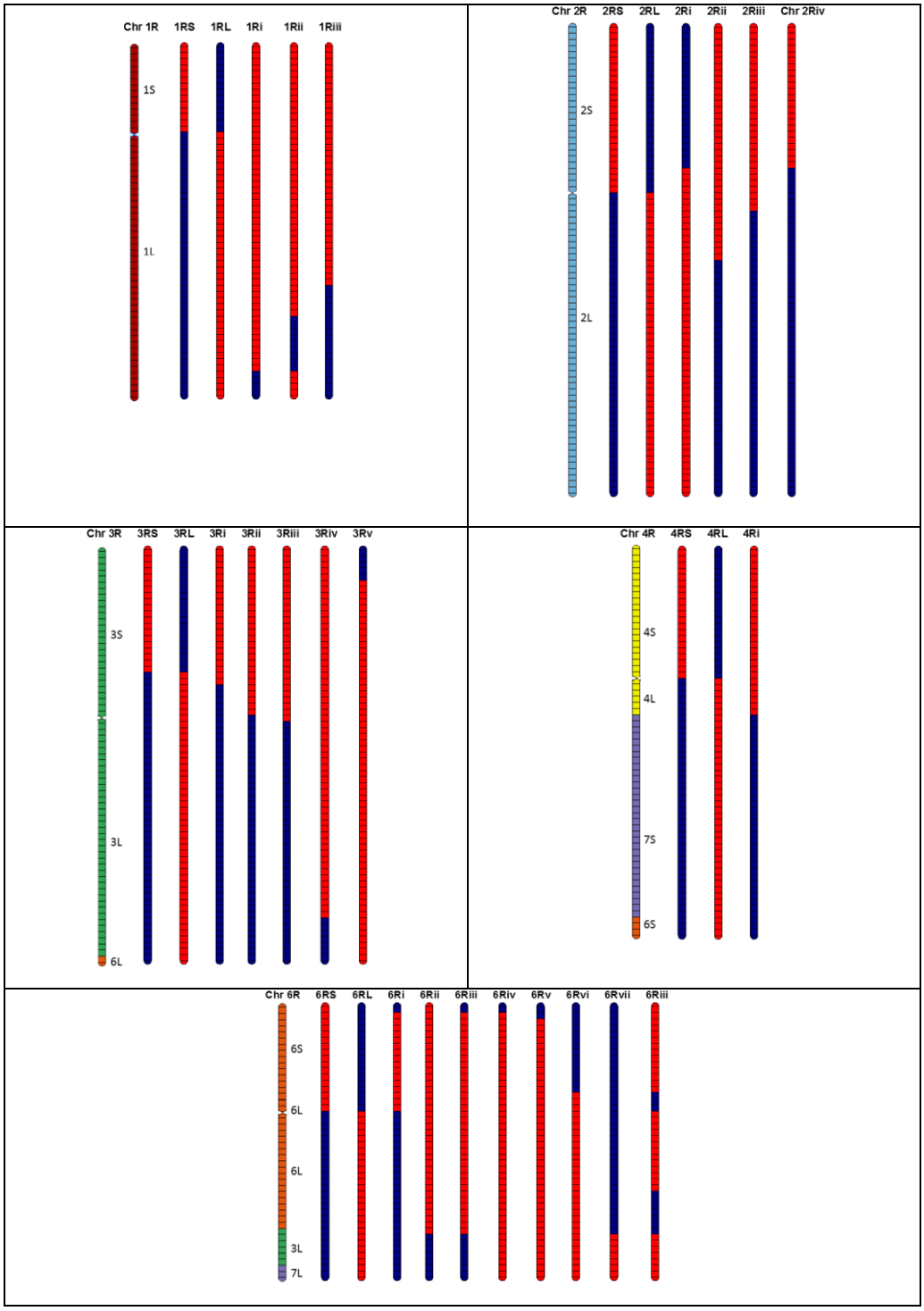
The mapping of chromosome 6R agrees with the mapping in Li *et al.* (2013), including the presence of a region of 6L proximal to the centromere in 6RS that was not reported in Devos *et al.* (1993)

7R

The mapping of chromosome 7R is mostly in agreement with the mapping in previous studies. However, compared to the previous studies the map

reported here has a smaller region of terminal 2S on 7RL. This is again likely to be due to a lack of marker coverage at the terminal regions of 7RL. The study also reports a region of 7S present proximal to the centromere in 7RS, agreeing with the map produced by Li *et al.* (2013).

Putatively this work shows a relationship between the genome of structure of rye and the type and size of wheat-rye introgressions. Figure 5.13 compares the genome orthologous genome structure of rye compared to wheat (figure 5.4) with the introgressions produced by this work and detected by the Axiom wild relative SNP array. The size and position of introgressions seems to be limited by the genome rearrangements present within rye. The evidence is two-fold; firstly, the linkage groups with less extensive rearrangements (1R, 2R, 3R and 6R) have more introgressions of varied sizes compared to the linkage groups with more extensive rearrangements (4R, 5R, and 7R) that have less introgressions. Secondly the position of the genome rearrangements correlates with the point of recombination/fragmentation in 6 of the introgressions. This relationship is most likely due to the presence of genome rearrangements restricting recombination as the chromosomes with rearrangements will not align properly with chromosomes without rearrangements therefore a recombination site will not be formed.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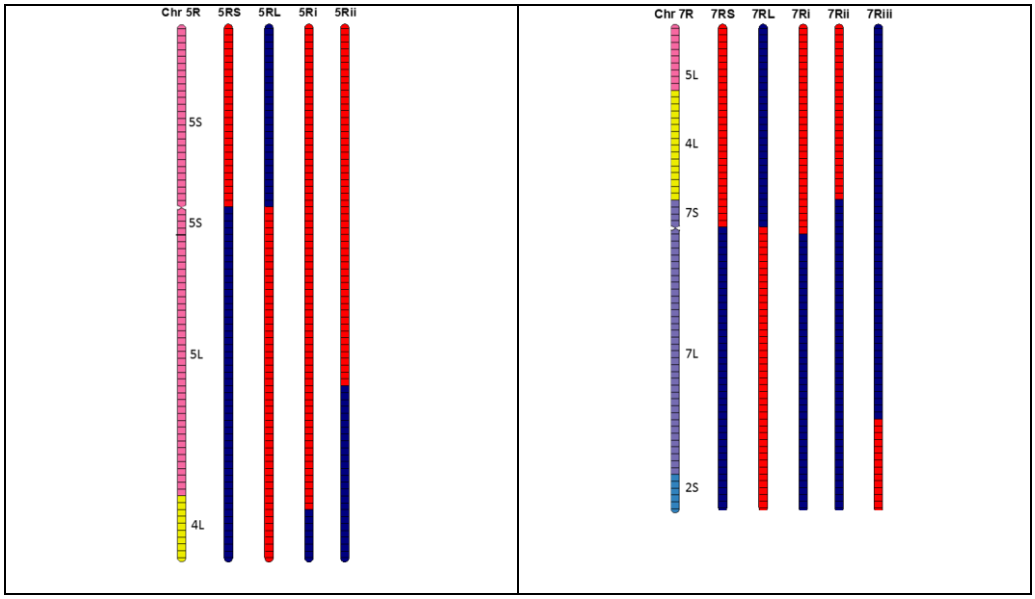

Figure 5.13. A representation of each of the chromsomes of rye and the translactions present as shown in figure 5.4 aligned to the wheat-rye introgressions by the Axiom wild relative array. The first rye chromosome in each box is coloured accroding the the orthologous wheat region it maps to, the remaining chromosomes are coloured according the introgressed regions of rye with red representing rye alleles and blue representing wheat alleles.

SNP platform and further development

The Axiom SNP array allowed lines to be genotyped by a substantial number of SNP markers. However, only 1.5% of the markers on the array were successfully incorporated into the physical bin map. The low conversion of markers on the array into the map may be due to several reasons: Firstly, the markers on the array were designed for a range of wild relatives meaning some of the markers did not work with rye. Secondly, criteria for PHR markers (polymorphic and codominant with a minimum of two examples of the minor allele) caused the largest reduction in marker number. The PHR markers were considered to be the best markers and therefore this reduction in marker number was considered justified. Markers needed to be homozygous in wheat and homozygous but different in rye. Thirdly, further analysis of some of the SNP data (not presented) suggested that a significant proportion of the SNPs included on the Axiom array were not polymorphic and hence this also caused a considerable reduction in the number of useable SNPs.

Recent development of both the wheat and rye genome sequences (Bauer *et al.* 2017; Zimin *et al.* 2017; IWGSC 2018) will expedite marker design. With the declining costs of genome sequencing and improvements in bioinformatic techniques, it is likely that the genome assemblies of other important wild relative species will become available/financially viable to produce, therefore, aiding the development of marker systems to use in introgression programmes in a wider range of wild relative species.

The limitations of this genotyping and work moving forward

The information that can be gathered about each introgression using this technique is limited. The polyploid nature of wheat means there is a copy of most markers within each of wheat's three genomes, i.e. each marker is not genome specific. Therefore, the presence of rye chromatin can be determined but it is not possible to tell if an introgression is the result of

recombination or the addition of a chromosome/chromosome aberration. The presence of multiple genomes also means this type of marker cannot indicate if a region of wheat chromatin has been lost and therefore, it cannot determine the location of a recombination event or whether the introgression is heterozygous or homozygous.

The F_1 lines genotyped all showed a complete set of rye chromosomes as was expected in the crossing scheme. Genotyping the F_1 plants was useful as a control to test the map and to also confirm that the F_1 plants had been produced correctly. However, the genotyping of the F_1 plants did not help in further identifying introgression lines as introgressions are first found in the BC_1 plants due to recombination occurring in the gametes of the F_1 plants. Furthermore, most of the BC_1 plants also showed complete coverage of rye chromosomes and therefore SNP genotyping at this stage was largely uninformative. Thus, most genotyping was carried out once the BC_2 generation had been reached.

5.5 KASP Background

A simplified explanation of the KASP system

To produce a more informative SNP genotyping platform, markers used in the axiom array are currently being converted to genome specific Kompetitive Allele Specific PCR (KASP™) markers [http://www.lgcgenomics.com, (Semagn *et al.* 2014)]. KASP™ genotyping is an PCR based system that is measured at its end point, during the PCR reaction two allele specific forward primers are used with a non-specific reverse primer. The two forward primers are competitive and the primer with the correct allele will out compete the alternative. The tail sequence of both forward primers contains an allele specific sequence that is complementary to a universal FRET (fluorescence resonant energy transfer) cassette and the tail sequence is incorporated into the PCR product. The FRET cassettes are labelled with allele specific fluorescent dyes (HEX™ or FAM™) and will fluoresce if able to bind to a tail sequence. Therefore, as the PCR progresses the amount of allele specific tail sequence increases allowing the FRET cassette to bind to the DNA and therefore a fluorescent signal that is dependent on the allele(s) present is emitted.

KASP genotyping aims

The KASP genotyping aims to produce a set of genome specific markers that will be able to identify introgressions as well as detecting if an introgression is homozygous or heterozygous, by determining if the respective wheat chromatin has been lost. This further allows identification of the wheat chromosome involved in the recombination event.

5.6 KASP Materials and Methods

SNP detection

To confirm SNPs between wheat and wild relatives, markers were chosen from the Axiom® 35K Wild -Relative Genotyping Array, and PCR was used to amplify the marker region in wheat and the 10 wild relatives. The resulting

PCR products were sequenced and compared to insure the presence of a wheat wild relative SNP and the sequence data was used to design KASP markers. The method is described in more detail below.

The markers from the Axiom® 35K Wild -Relative Genotyping Array that were predicted to have a single copy in wheat based on the Axiom 820K Array data (Winfield et al. 2016) were selected and these covered all seven-wheat homologous groups. The sequence of the chosen axiom markers was compared to the wheat genome assembly IWGSC RefSeq v1.0 (IWGSC 2018) using BLAST with a cut off e-value of 1e-05. From the BLAST results the corresponding wheat sequence from all three genomes was taken if available. These results were used to a) assign markers their corresponding physical positions on the wheat chromosomes, b) ensure good coverage over the whole wheat genome and c) check marker alignment against the genomic sequence to ensure the SNP was not between exons or at the end of the sequence.

The sequence with the highest BLAST score was taken and used to design PCR primers using Primer3 (web version 4.0.0, available at http://primer3.ut.ee/) that cover a 100-300 bp either side of the targeted SNP to produce a total product length of 500- 550 bp. The primers were produced by Eurofins UK and de-ionsed water was added to produce working solutions at the required concentrations.

PCR was performed with the primers designed above, on the total genomic DNA (section 2.3) from wheat, rye and the other nine wild relatives (*Ae. caudata, Ae. speltoidies, Am. muticum, Th. bessarabicum, Th. elongatum, Th. intermedium, Th. ponticum, T. urartu and T. timopheevii* [as part of a wider project to develop KASP markers for a larger selection of potential wild relative gene donors]). The PCR products where then run on a 1.6% agarose gel and products that were the correct size were extracted from the gel and cleaned using NucleoSpin® Gel and PCR Clean up kits (Macherey-

Nagel). The extracted PCR products were sent for sequencing by Source Biosciences (http://www,sourcebioscience.com/). The resulting sequences were aligned using GeneDoc (version 2.7) to check for SNPs between wheat and all ten wild relative species including rye. The sequences where also checked for alignment to determine the presence of any large insertions or deletions and to remove any column gaps in the sequence. After SNP discovery, a 50 bp sequence on either side of the SNP was chosen and the SNPs were replaced with their IUPAC code (IUPAC SNP nomenclature) in the Chinese Spring sequence with the target SNP also marked with square brackets. If possible, marker regions with only a single SNP were used.

The marker sequence was then used in a final BLAST search against the wheat reference genome sequence RefSeq v1. The advanced parameter was set to (-e value 1.0e-10-max_target_seq_3) and the base pair scaffold position was recorded if the results had a BLAST score over 90. If a sequence gave hits on multiple chromosomes their positions were also noted, to determine if a marker was chromosome specific. A panel of 1000 markers were selected spread across all 21 wheat chromosomes to be converted to KASP assays. Chromosome specific markers were obtained in two ways firstly if a marker sequence was present in only one wheat genome and the rye genome the marker is wheat genome specific, secondly if the marker sequence is present in multiple wheat genomes but contains a wheat genome specific SNP that was the same in the rye genome the reverse primer was designed to ensure genome specificity, see fig 5.14 and 5.15. Figure 5.16 shows the how genome specific markers are used to indicate the presence of homozygosity in an introgression line.

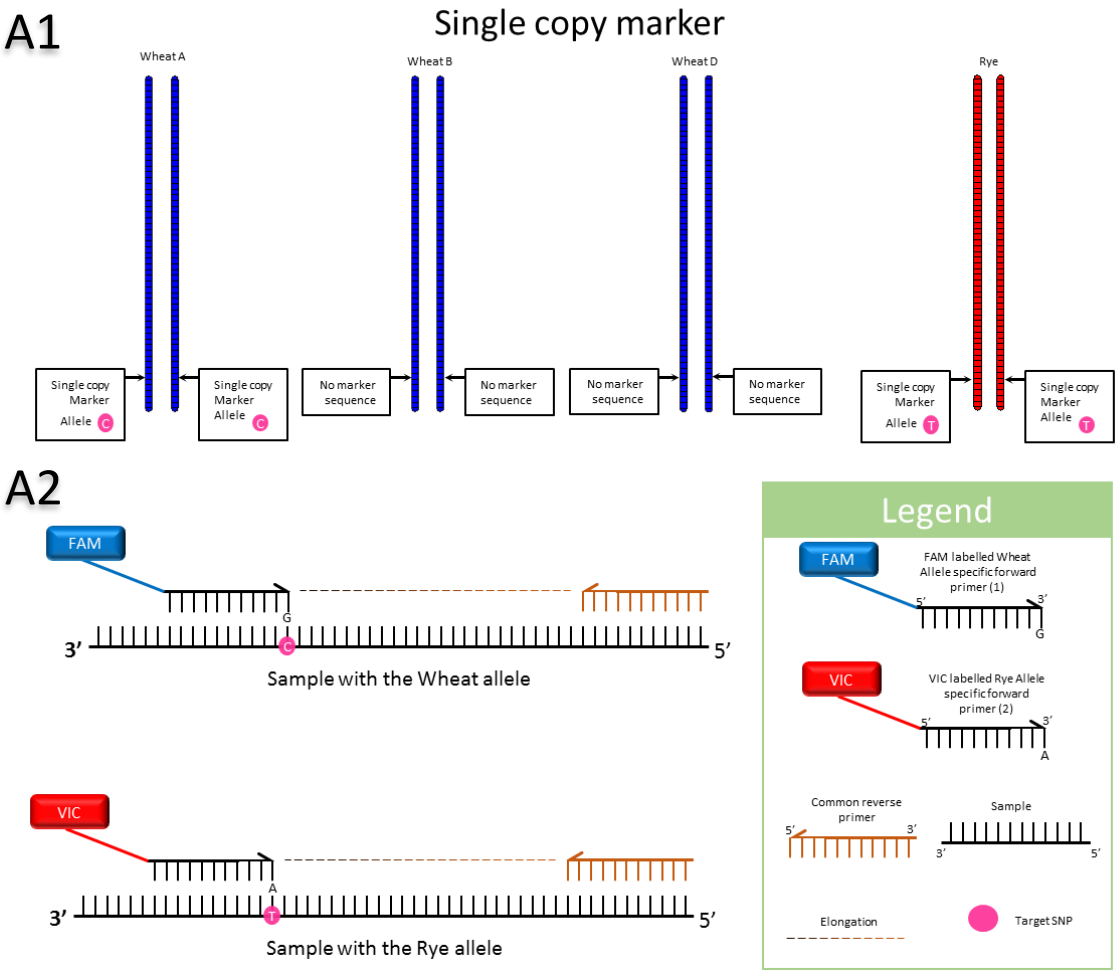

Figure 5.14 How genome specificity is insured in single copy KASP markers. A1 represents chromosomes from one homoeologous group, red indicates rye and blue indicates wheat the important SNP alleles are indicated. A2 shows the binding of the labelled Allele specific forward primers and the common reverse primers. Each forward primer is specific to an allele and carries the binding site for a KASP fluorescently labelled cassette.

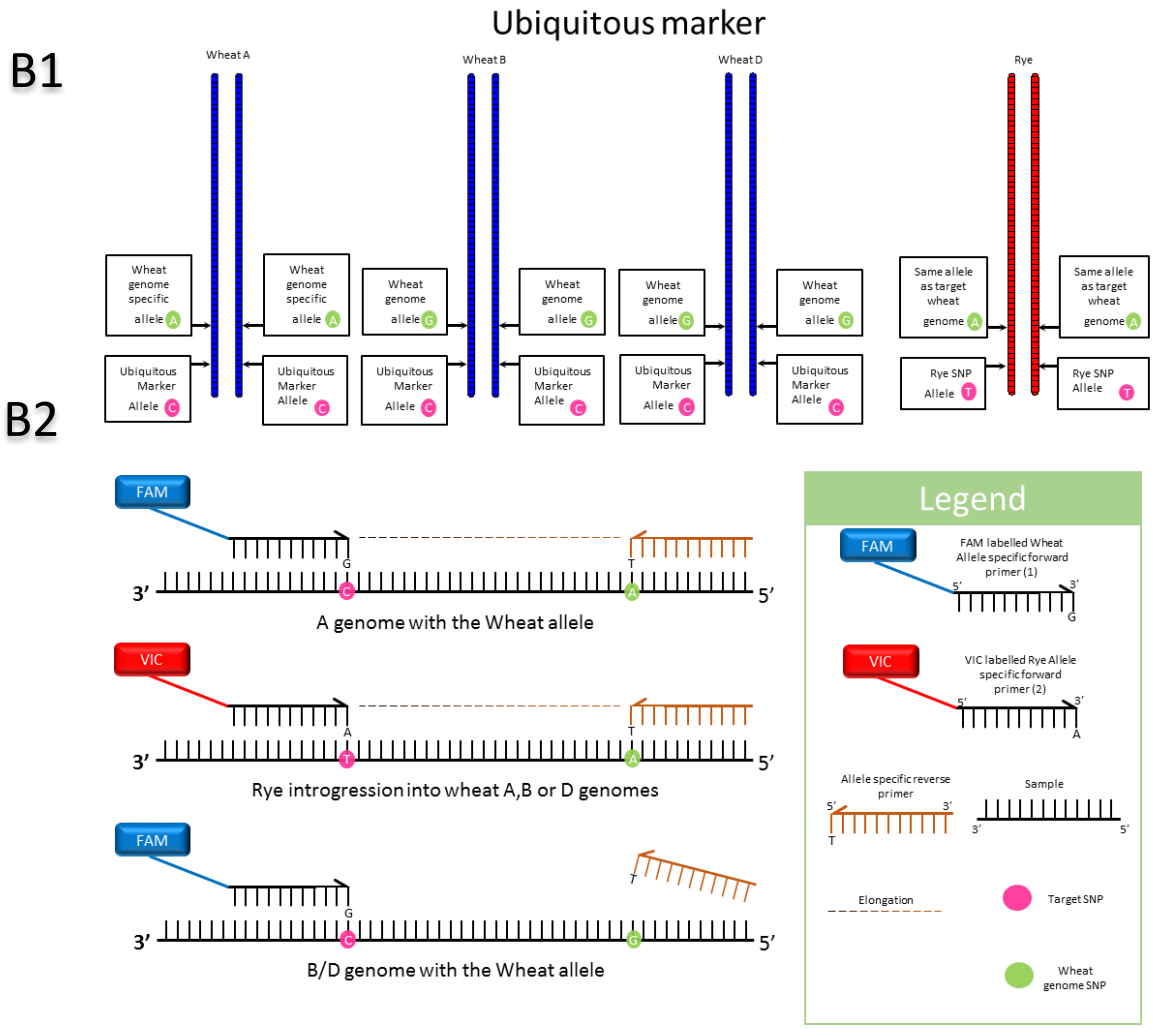

Figure 5.15 How genome specificity is insured in KASP markers with multiple copies on differing wheat genomes. B1 represents chromosomes from one homoeologous group, red indicates rye and blue indicates wheat the important SNP alleles are indicated. B2 shows the binding of the labelled Allele specific forward primers and the allele specific reverse primer. The target allele in the reverse primer is only present in the target wheat genome and the rye genome. Each forward primer is specific to an allele and carries the binding site for a KASP fluorescently labelled cassette.

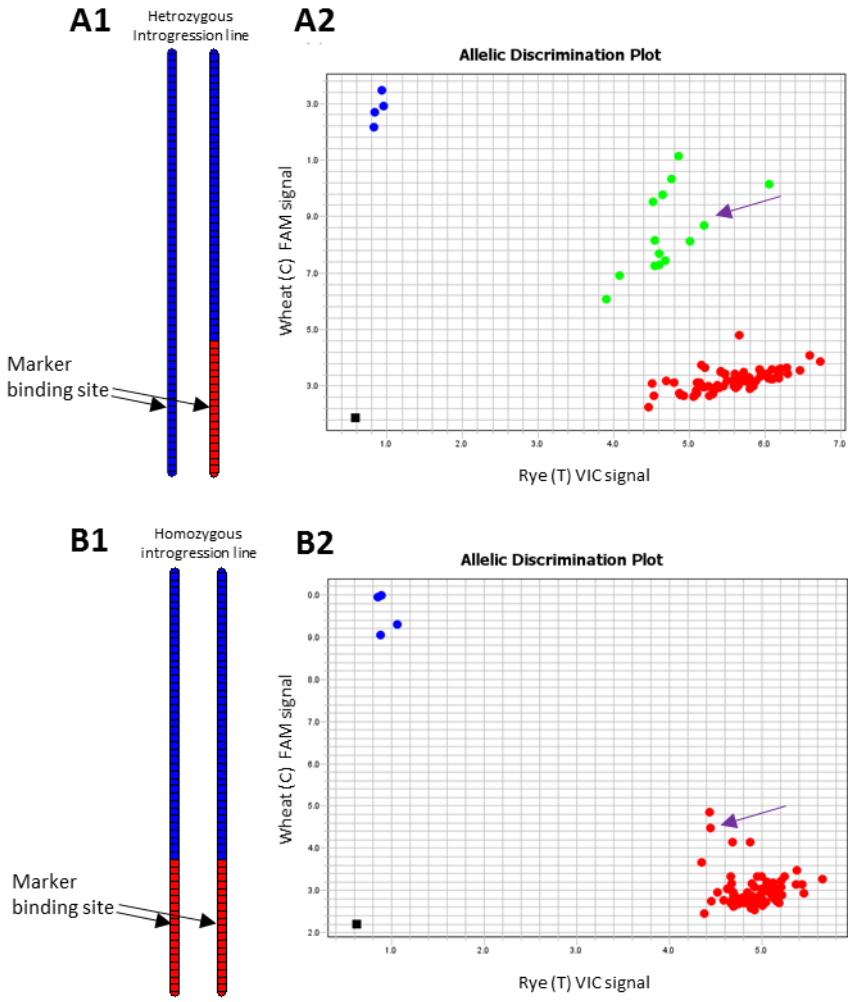

Figure 1.16 KASP Allelic discrimination plots indicating the presence of a heterozygous and a homozygous rye introgression. **A** show a heterozygous introgression and **B** shows a homozygous introgression. **A1** and **B1** represent chromosome pairs, the red regions represent introgressed rye chromatin and blue regions represent wheat chromatin. **A2** and **B2** show the KASP allelic discrimination plot for the same marker (the binding site is indicated). Each coloured circle is a different sample the green circles are homozygous wheat and include the wheat controls, the red samples are homozygous rye and contain the rye controls, the green samples are heterozygous introgressions and therefore have both signals. The purple arrow in **A2** depicts the sample shown by **A1**. The purple arrow in **B2** depicts the sample shown by **B2**

It should be noted that the KASP assay design work including all the PCR primer design, amplification, clean-up and sequence analysis was done in collaboration with other members of the WRC group. All PCR was performed on all 10 wild relatives not just rye. Moreover, SNP discovery resulting from my PCR work was done for all wild relative species. The groups contribution was pooled to obtain a panel of 1000 markers that covered all 21 chromosomes of wheat and different markers were polymorphic for between wheat and all 10 wild relative species (unpublished data).

The marker sequences along with 5,000 DNA samples from the whole wild relative programme (including 467 rye lines) were sent to LGC for KASP assay design and genotyping. The rye lines sent to LGC consisted of the rye accessions 390382 and 428373, the available Imperial rye addition lines 1R, 2RS, 3R, 4R, 5R, 5RL, 6R and 6RL (confirmed using GISH as described above) and 457 experimental lines produced by the crossing scheme described in chapter three. The genotyping data received was analysed alongside the wheat lines Chinese Spring, Highbury, Paragon and Pavon 76 as controls.

Genotyping analysis

Markers were deemed to be effective if the wheat and rye controls showed a homozygous call for different alleles. Any marker that showed the same call for all introgression lines was also disregarded. The additions lines were used as a control to confirm that markers were working on the intended homologous group. If a marker sequence was suspect, the raw data was checked using SNP viewer (snpviewer.sourceforge.net) to identify it in the clusters.

The KASP data was analysed in two ways using two different marker orders. First, the markers were divided into the 21 wheat chromosomes for which they were designed and ordered using their corresponding physical base pair position on the wheat genome. Secondly, markers were ordered

according to the rye chromosome structure (with chromosomal translocations compared to the wheat genome) using the wheat physical position of the KASP markers and aligning them according to where they fall within the bin map of rye (Figure 5.2). Nineteen lines were genotyped with both the axiom array and KASP markers and these lines where compared to validate the KASP markers.

Detecting homozygosity was possible because each marker was chromosome specific. Homozygosity was inferred by the presence of a rye allele and the complete loss of the corresponding wheat allele, from one wheat genome. For example, in a 1RL-1AL homozygous introgression, 1RL is present and part of 1AL has been lost, therefore, markers covering the introgression on 1RL, will only show the presence of the rye allele as a homozygous call. In addition, markers for 1BL and 1DL corresponding to the 1RL introgression will detect its presence as heterozygous because both the rye allele and the B and D genome wheat alleles will be present. It was also possible to detect when a wheat chromosome has been lost, but not replaced by a rye introgression, as the markers for wheat chromosome shows no signal, while the markers from the homoeologous wheat chromosomes show wheat alleles.

Figure 5.17 illustrates how the KASP markers detect to position of a homozygous introgression and shows where in each of the four possible genomes present (three wheat, one rye) the genome Specific KASP markers bind

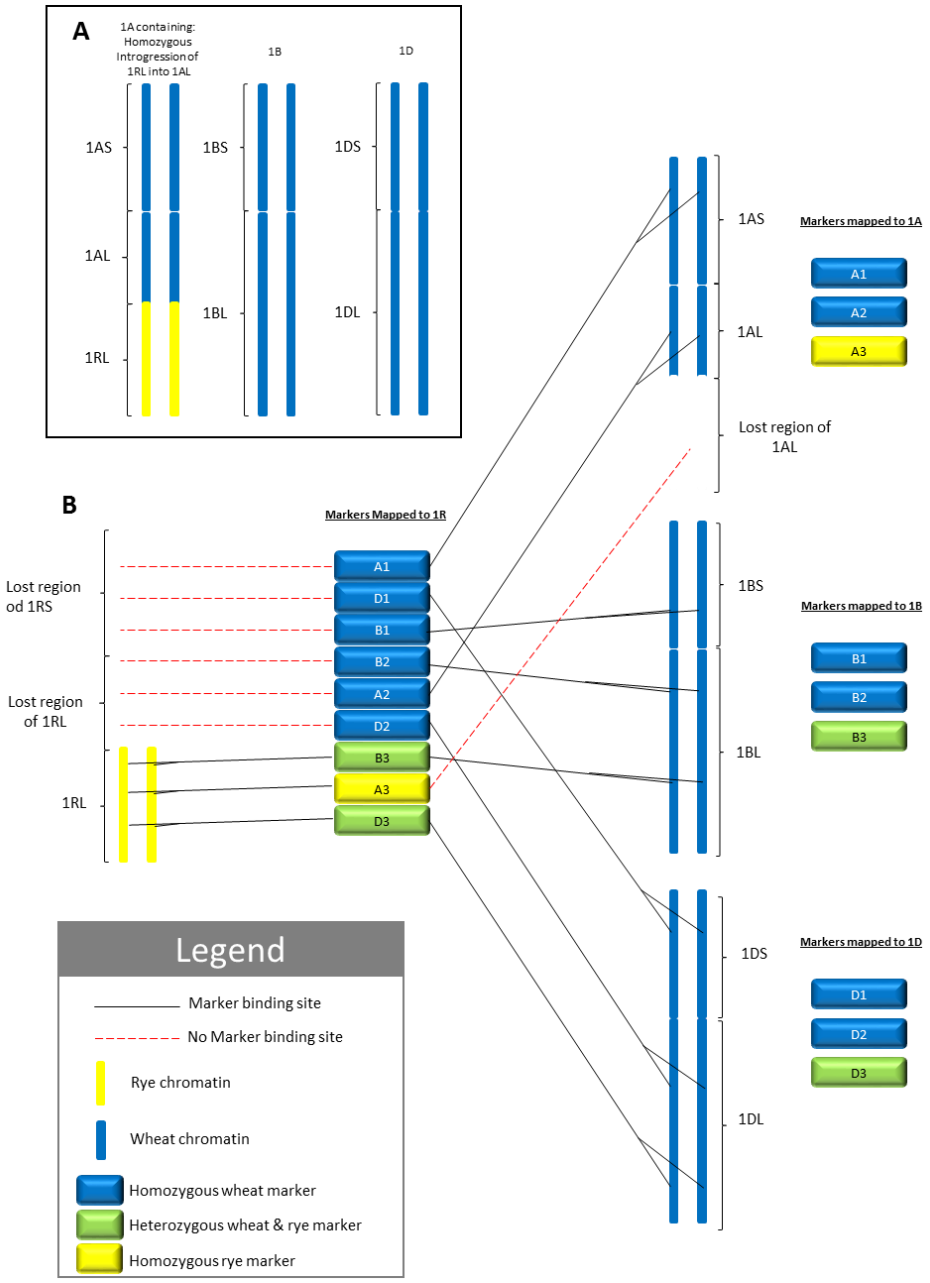

Figure 5.17 An illustration of KASP marker mapping and interpretation. **A** shows the chromosome composition in homoeologous group 1, of the line tested by the markers indicated below. Example line contains a homozygous introgression of 1RL into 1AL. Blue bars indicate regions of wheat chromatin and yellow bars indicate regions of rye chromatin. **B** shows how the KASP markers detect the presence of each genome, the markers are shown by the coloured boxes, the markers are ordered in two ways, on the far right the markers are separated into each wheat genome and in the centre of the figure the markers are ordered according to the rye genome. The sites of each marker allele (only shown on markers ordered according to the rye genome for simplicity) is indicated by a line, whole black lines indicate the alleles binding site is present were as red dashed lines show a lack of the binding site of a specific allele. Markers that only have wheat allele binding sites present are blue and indicate only wheat. Markers that have a rye allele binding site present and wheat allele binding site present are green and indicate the presence of both wheat and rye. Markers that only have a rye allele binding site present are shown in yellow and indicate the presence of homozygous rye and the loss of the corresponding wheat region

5.7 KASP Results

Marker efficacy and order

A total of 1000 markers were designed to work across all 21 chromosomes of wheat and be polymorphic with all 10 wild relatives. Of these, 143 KASP markers were designed to work on rye with scope for more markers to be successful on rye out of the remaining 857 markers. After genotyping it was found that 124 (12.4% of all markers) were shown to effectively identify the wheat and rye controls. A breakdown of the number of markers designed and working per homologous group is shown in table 5.3.

Table 5.3. The number of KASP markers designed to identify rye chromatin over the 7 homologous groups of wheat and the number of markers working on rye, after the testing of all 1000 markers.

Linkage group	Designed for rye	Working
1	25	27
2	23	22
3	18	18
4	24	15
5	18	17
6	17	14
7	18	11

After the initial testing on controls, the marker numbers were further reduced when re-ordering markers to reflect the structure of the rye genome and during comparison to the 19 lines that had been previously genotyped using the axiom array. In total, 88 markers were used after the marker order had been finalised and checked against pre-existing genotypes. The final number of markers per rye-homologous group are show in table 5.4

Table 5.4. The number of KASP markers in the final order in using rye homologous groups

Rye Homologous group	Number of markers
1	15
2	15
3	9
4	8
5	15
6	18
7	8

Comparison of Lines genotyped with Affymetrix and KASP

There were 19 lines that were genotyped with both KASP and the Axiom array. Figures 5.18 through 5.24 show GGT images comparing the KASP and Axiom genotypes of the 19 lines for each rye homologous group. In general, the two genotyping methods concur with some differences due to marker number, distribution, and resolution.

Groups 1R and 2R completely agreed across both genotyping platforms with the only differences being due to the differences in the marker number skewing the size of the introgressions (Figure 5.18 and Figure 5.19).

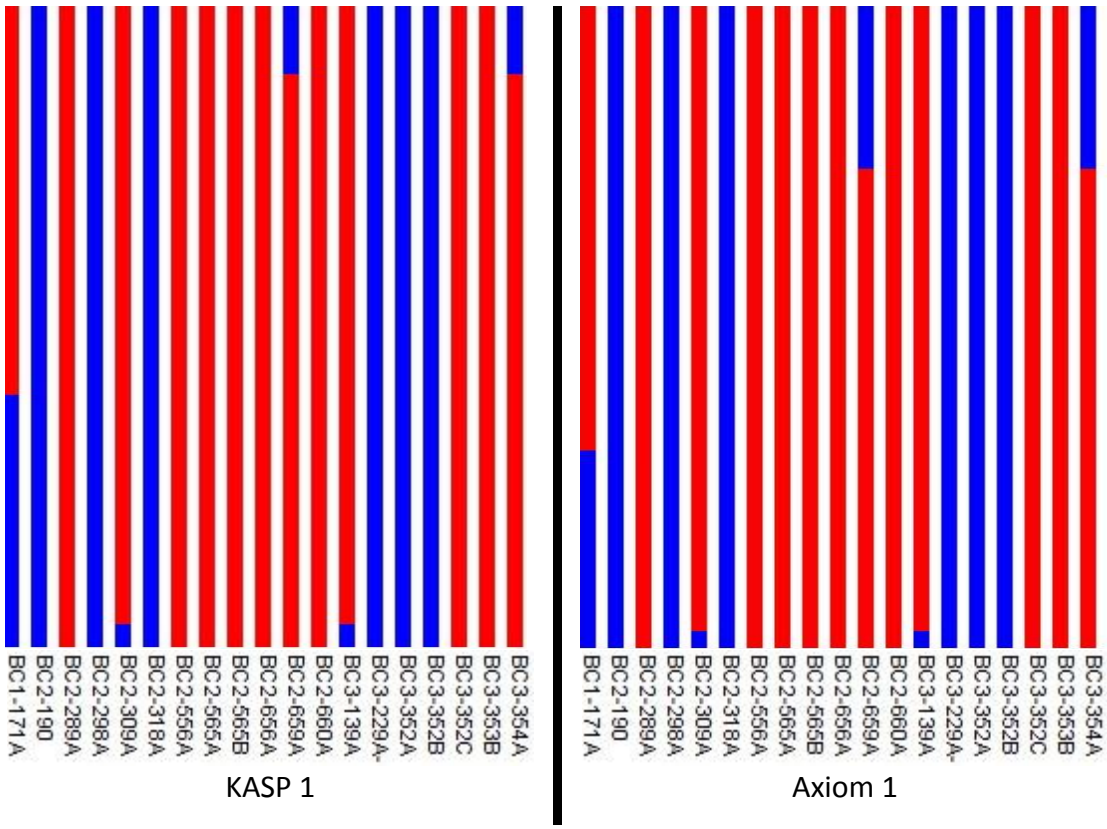

Figure 5.18 The 1R genotypes of 19 introgression lines from KASP and the Axiom array visualised with GGT2.0 (van Berloo 2008)

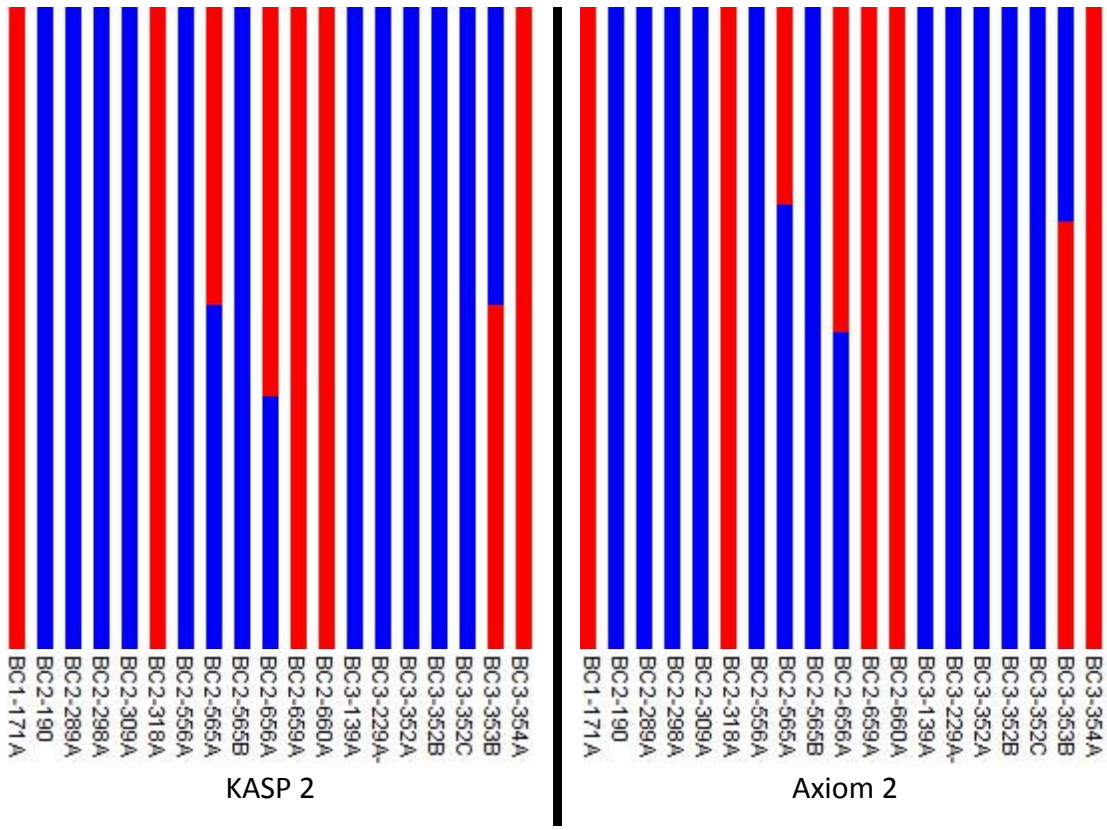

Figure 5.19 The 2R genotypes of 19 introgression lines from KASP and the Axiom array visualised with GGT2.0 (van Berloo 2008)

Group 3R generally agrees across both genotyping platforms with the KASP platform showing a lack of resolution around the centromere and at the terminal region of 3RS demonstrated by the genotype of BC2-565B (Figure 5.20

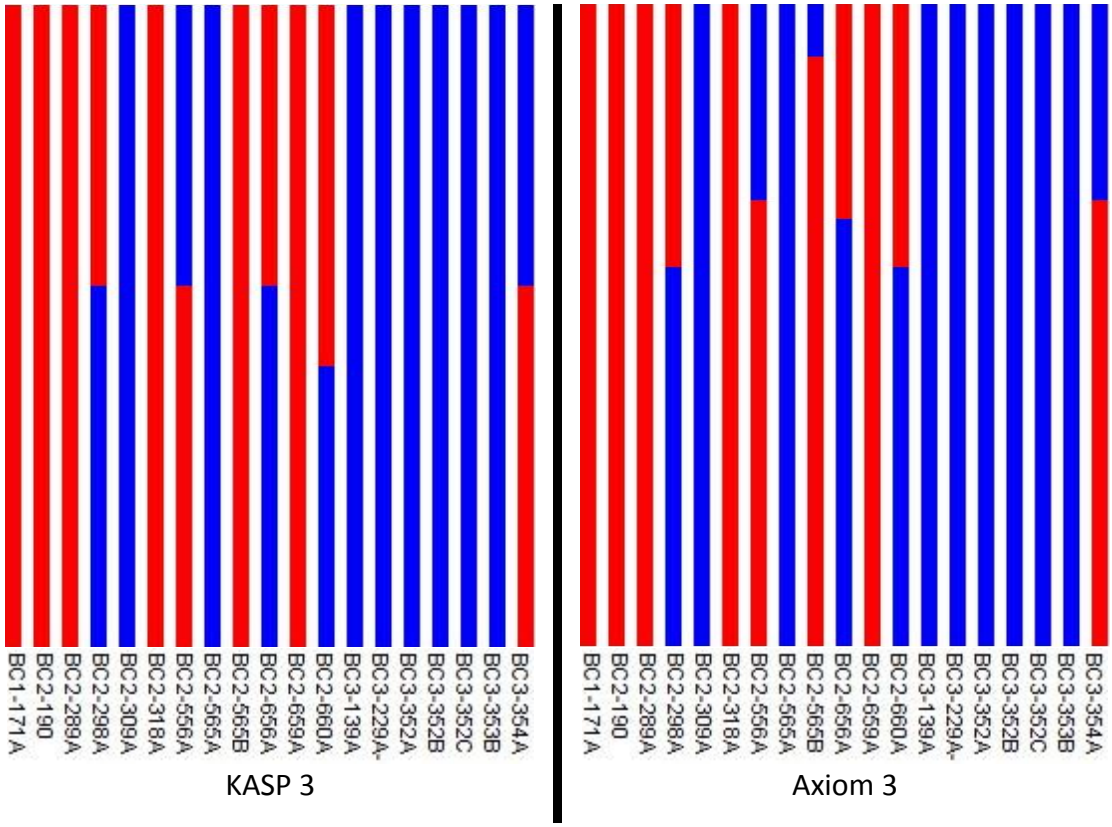

Figure 5.20 The 3R genotype of 19 introgression lines from KASP and the Axiom array visualised with GGT2.0 (van Berloo 2008)

Group 4R agrees across both genotyping platforms with the KASP platform showing a lower resolution around the centromere, figure 5.21.

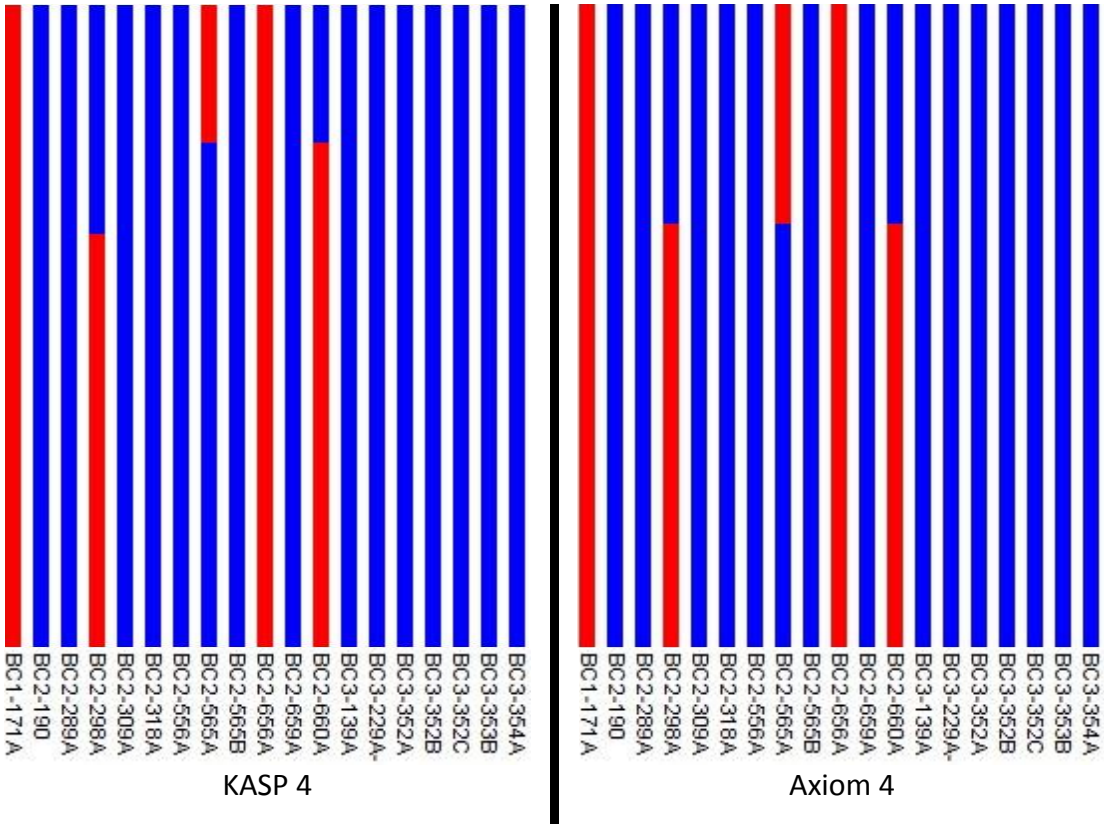

Figure 5.21 The 4R genotype of 19 introgression lines from KASP and the Axiom array visualised with GGT2.0 (van Berloo 2008)

Group 5R generally agrees across both genotyping platforms with some differences being due to the differences in the marker number skewing the size of the introgressions. KASP genotyping shows a distal wheat region on the 5R short arm of BC2-565B and BC2-660A that is not present in the Axiom array (Figure 5.22).

Group 6R also agrees in both genotyping platforms, except for a terminal wheat region on the short arm in BC3-352A that is not present in KASP but is in the Axiom genotype (Figure 5.23).

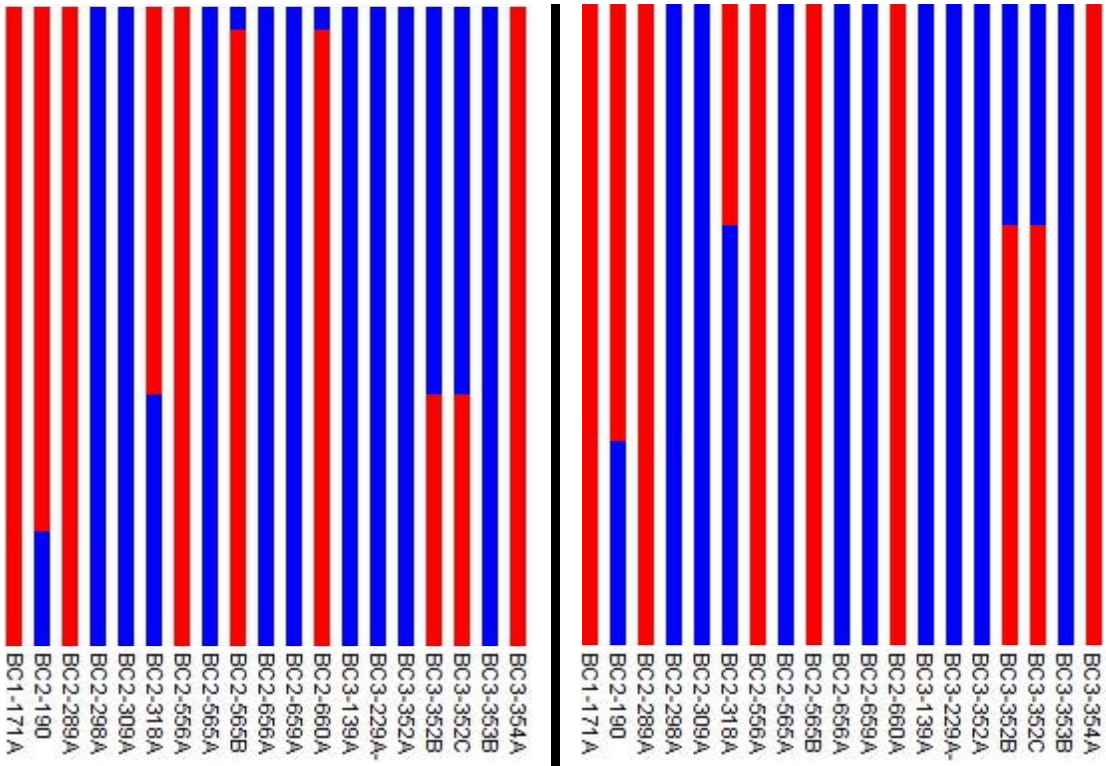

Figure 5.22 The 5R genotype of 19 introgression lines from KASP and the Axiom array visualised with GGT2.0 (van Berloo 2008)

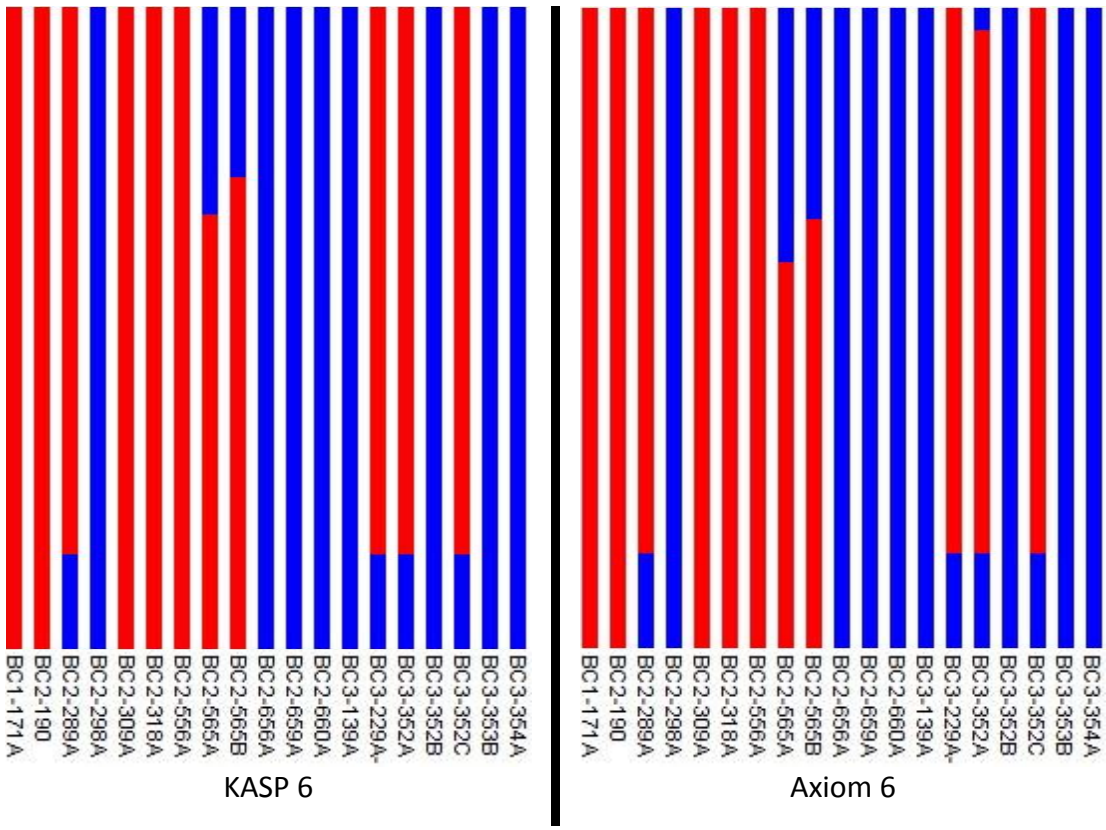

Figure 5.23 The 6R genotype of 19 introgression lines from KASP and the Axiom array visualised with GGT2.0 (van Berloo 2008)

In group 7R, both genotyping platforms agree, with KASP showing a slightly lower resolution at the centromere.

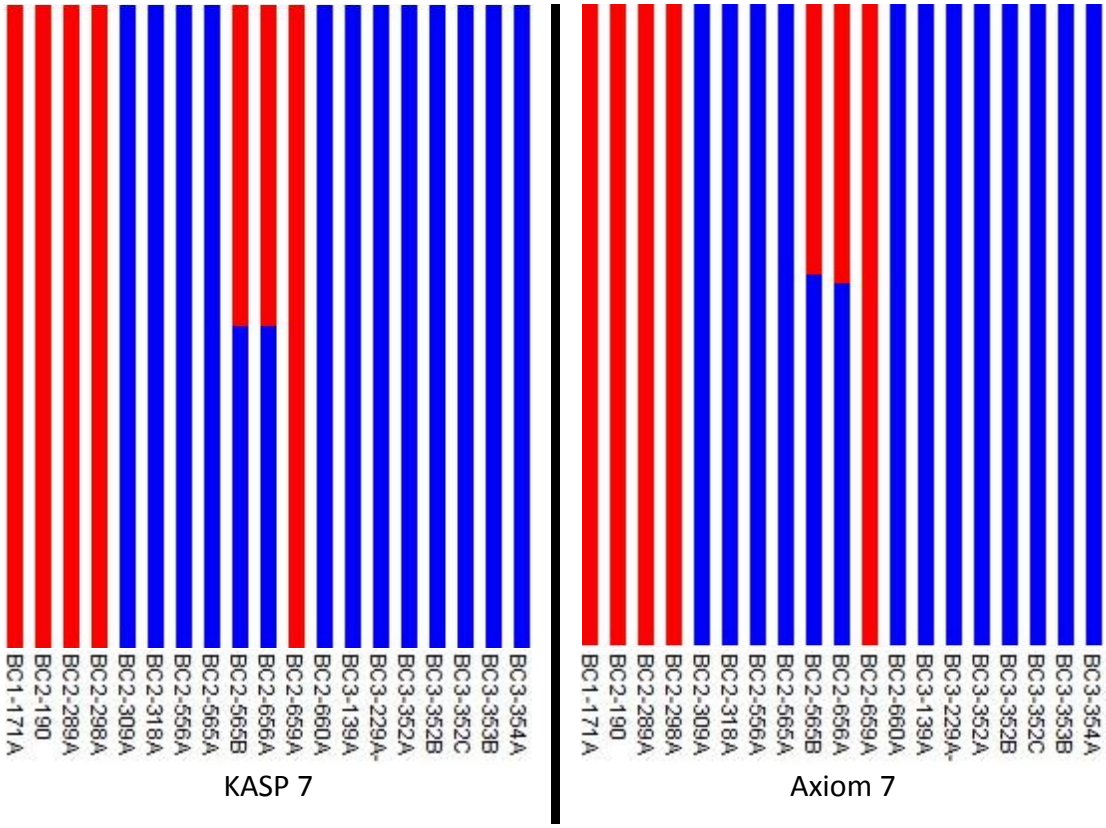

Figure 5.24 The 7R genotype of 19 introgression lines from KASP and the Axiom array visualised with GGT2.0 (van Berloo 2008)

Identification of introgressions with KASP

Introgressions were identified using the markers ordered according to rye chromosome structure. A total of 513 introgressions were identified with 201 of the introgressions being whole chromosomes and 53 unique introgressions. A summary of the distribution of introgressions from each rye chromosome is shown in table 5.5. The KASP results showed lines with whole chromosome introgressions in every rye linkage group, as well as smaller introgressions representing every separate rye chromosome arm, including lines with 6RS that was not present in the lines genotyped with the axiom array.

Table 5.5 A summary of the introgressions identified using KASP, the total number of introgression lines per rye homologous group is shown as well as the number of whole chromosome introgression

Rye Chromosome	Number of lines containing introgressions	Number of lines containing whole chromosome introgressions	Unique introgressions
1R	129	65	10
2R	55	19	9
3R	65	23	6
4R	32	8	5
5R	74	25	9
6R	105	43	8
7R	53	18	5

As mentioned above, due to the structural differences between wheat and rye it was necessary to use two marker orders. The first was separated into each of the 21 wheat chromosomes and ordered according to their physical positions to enable the detection of lost wheat chromosomes. The second genotyping was with the markers separated into the seven rye chromosomes and ordered according to their physical positions in respect to the rye genome, to indicate the region of introgressed rye chromatin.

For example, in the introgression line BC_2F_1-255D, the KASP data ordered according the rye shows an introgressions 1R, 5RS, 6RL and 7RL. The KASP data for the homologous group 1 markers, ordered according to wheat showed a majority of heterozygous markers for 1A and 1D, and homozygous rye markers for 1B. When combining the KASP data the GISH image from BC_2F_1-255D and the axiom, KASP and GISH data from its parent it was possible to determine that the line contains a homozygous 1R (1B) substitution, a 7RS.6RL centric fusion and a 5RS centric fusion with an

undetermined wheat chromosome. It was possible to say that 5RS had fused with wheat because the centric fusion was present in previous generations, whereas 7R and 6R were whole chromosomes implying that in the gametogenesis in the parental plant 7R and 6R have broken and the 7RS and 6RL have fused, with the remainder of these rye chromosomes being lost. Figures 5.25 and 5.26 shows the KASP data and a GISH image of line BC_2F_1-255D.

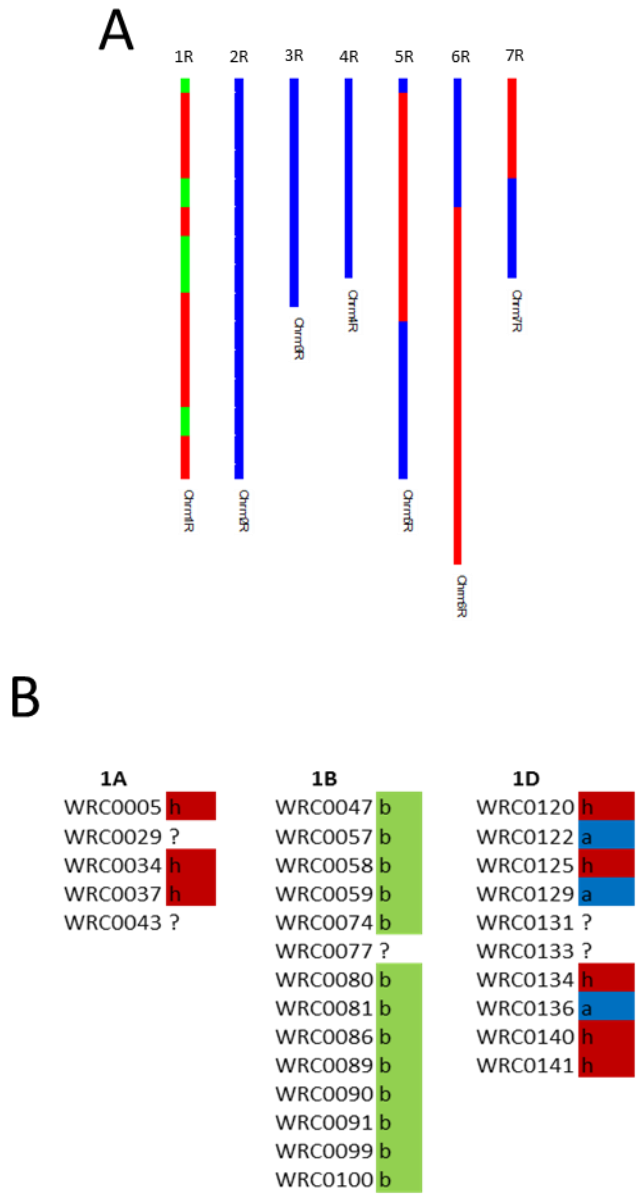

Figure 5.25. Characterisation of BC_2F_1-255D using KASP™ **A** shows the KASP marker calls, ordered according to the rye genome, from every homoeologous group and visualised with GGT 2.0 (van Berloo 2008) The regions of red show heterozygous rye markers, green are homozygous rye makers and blue is homozygous wheat. The results from A show a whole homozygous 1R introgression (indicated by the green region) but does not show which wheat chromosome 1 has been lost. A also shows the presence of large regions of 5R, 6R and 7R

B shows the markers from homoeologous group 1 and they are ordered according to the wheat chromosomes 1A, 1B and 1D markers are coloured as in A. The results from B show that the Homozygous 1R introgression is a 1R-1B substitution.

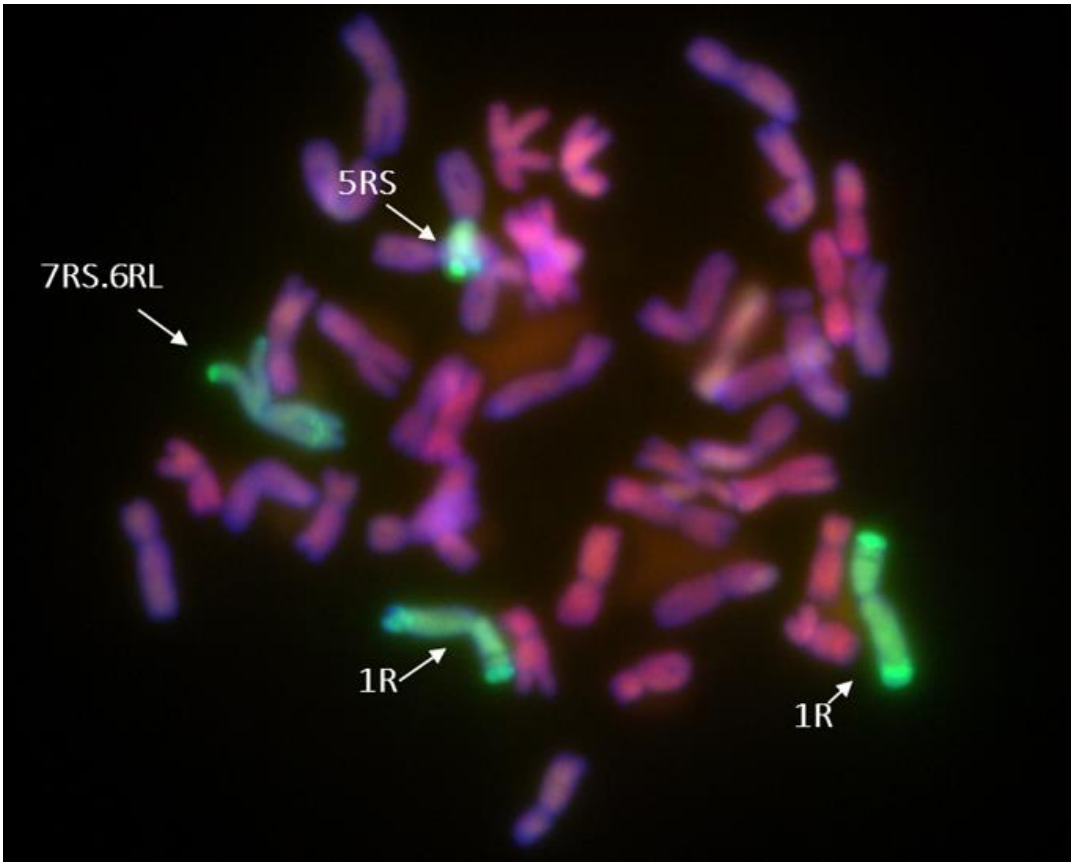

Figure 5.26 Characterisation of BC_2F_1-255D using GISH. A single colour GISH image of the same line shown by figure 5.25. Rye chromatin has been probed using genomic rye DNA and shown in green and wheat chromosomes are shown in blue. The GISH image alone shows the presence of three whole rye chromosomes and a wheat-rye translocation

By combing the KASP results (figure 5.25) the GISH image above and the same information gather from the parental plants of BC_2F_1-255D.It was possible to identify each of the introgressions. The results show BC_2F_1-255D contains a whole homozygous 1R:1B substitution a 7RS.6RL centric fusion and a 5RS centric fusion with an unknown wheat chromosome.

41 lines were identified with a minimum of one marker showing a homozygous introgression. A summary of the lines showing homozygous lines and their most likely composition is shown in table 5.6.

Chromosome 1A showed five lines from the same family with homozygous regions of 1A. Three of these lines had a 1R(1A) substitution and two lines had a 1RS-1AS introgression. Chromosome 1B had three lines with a 1R:(1B) substitution, three lines where 1BL had been lost but a whole rye chromosome 1R is present, one line where 1BS had been lost and the whole of 1R was present and one line with a 1RL-1BL introgression. Chromosome 1D has three lines that show 1R(1D) substitutions.

Chromosome 2A had one 2R(2A) substitution line. Chromosome 2B had one line with a 2RL introgression with no chromosome 2B. Chromosome 2B also had five lines where a 2B marker from the end of the long arm had been lost indicating the presence of homozygous rye chromatin. These markers map to the end of 7RL and are thus likely to be due to a distal 7RL-2BL recombinant. The mapping of chromosome 7R is relatively poor and therefore this is still uncertain. Chromosome 2D has one 2R(2B) substitution line.

Homologous group 3 had no homozygous introgression lines.

Chromosome 4A had one line where the whole of chromosome 4A had been lost but with an introgression of 7RS that maps to the 7RS region which is orthologous to 4A and therefore the KASP results show a homozygous region of 4A. There were no homozygous chromosome 4B markers. One line was homozygous in chromosome 4D and was the result of a chromosome 5R(4D) substitution.

Chromosome 5A had four lines that showed the loss of chromosome 5A and the introgression of 5RL. Two lines were 5R(5B) substitutions. No lines had gone homozygous in 5D.

In chromosome 6A, one line had lost 6AL and contained a whole 6R chromosome. No homozygous markers where present in 6B. Chromosome 6D contains had nine lines that were all 6R(6D) substitutions.

No markers were confirmed as homozygous in 7R.

Table 5.6 A summary of the introgressions shown to be homozygous with KASP. If multiple lines show the same introgression the number is indicated in brackets.

Wheat chromosome	Substitutions Present	Translocations/ Recombinants
1A	1R:1A(5)	1RS:1AS(2)
1B	1R:1B(3)	1RL:1BL
1D	1R:1D(3)	-
2A	2R:2A	-
2B	2RL:2B	7RL:2BL
2D	2R:2B	-
4A	7RS:4A	-
4B	-	-
4D	5R:4D	-
5A	5RL:5A(4)	-
5B	5R:5B(2)	-
5D	-	-
6A	6R:6AL	-
6B	-	-
6D	6R:6D(9)	-

Relationship between homozygous KASP and Affymetrix results

The 1RS:1AS and 1RL:1BL translocations detected by KASP were also detected by the the axiom array as an introgression of the respective whole chromosome arms, but the axiom array could not indicate the position of the introgressions in the wheat genome.

The 7RL:2BL introgression was present in family that was not tested using the Affymetrix array.

The remaining introgression described in the Affymetrix results section two have also been tested using KASP and show comparable introgression sizes but these lines and their progeny have not yet gone homozygous and therefore no further information is available

5.8 KASP discussion

The axiom array and KASP genotyping results both agree with only slight differences that are found either around the centromere or at the terminal marker regions. The differences present can be explained by marker distribution and the lower number of markers present in KASP, resulting in a lower resolution genotype. Therefore, the KASP genotyping was less likely to resolve differences between similar sized introgressions. The region containing the centromere was estimated from the axiom mapping and then compared to the KASP genotypes, revealing that the majority of introgressions (excluding whole chromosomes) were equivalent to whole chromosome arms, with the proviso that due to the low marker numbers an accurate estimation of the size of introgressions was not possible. A more accurate prediction of size could be obtained by developing more markers. This is especially true for the 3R, 4R and 7R groups that have almost half the number of markers as the remaining rye groups.

Interestingly some markers were able to identify a rye allele once it had become homozygous but could not identify heterozygous alleles. It is likely these markers could be made to be effective with optimisation at the PCR level.

Designing markers to be genome specific was shown to be a powerful tool, especially with markers ordered according to both wheat and rye as they allow for the detection of introgression that have become homozygous. Homozygosity was investigated using markers separated into each of the 21 wheat chromosomes and ordered according to their physical positions because recombination was hypothesised to be most likely to occur between orthologous regions irrespective of structure, i.e. wheat 4L is most likely to recombine with its orthologous sequences even though that sequence is present on chromosome 7R of rye. Homozygosity was assumed from the loss of wheat signal and the results were then compared to the results shown by the markers ordered according to the rye genome which allows the genotype to identify which region of rye chromatin had been introgressed. However, if a wheat chromosome has been lost irrespective of the presence of an introgression homozygosity could be falsely assumed.

The KASP data showed very promising results, but the results do need to be confirmed with a higher density of markers across all chromosomes. This could also be achieved by cytogenetic analysis and especially by using FISH to karyotype the wheat lines that the KASP markers show are missing wheat chromosomes or chromosome segments.

Genotyping using genome specific markers, though more informative than using non-specific markers, still shows the following problems:

- When introgressions are still heterozygous, the genotyping is no more informative than when using non-specific markers.

- A rye introgression with a single copy, where both copies of the corresponding region of wheat has been lost, cannot be distinguished from a truly homozygous introgression.
- Two or more separate introgressed chromosome fragments are indistinguishable from regions of introgressed chromatin that have fused together, e.g. a centric fusion.
- When a polyploid species with multiple copies of the same genome is used a genome donor, it is possible multiple introgressions of the same homologous group are captured and would be in-distinguishable. Moreover, if the introgressions are different sizes it would be impossible to determine the size of both introgressions.

Therefore, the KASP genotyping results will benefit from confirmation with cytogenetic techniques.

Progression of the KASP genotyping

For future genotyping the Nottingham Wheat Research Centre is transferring to an in-house system. Initial testing has been undertaken using a subset of the markers (shown to be working by LGC) on a sample of 94 rye introgression lines. The initial testing was not successful. However, the system is still in the process of being optimised. For example, the DNA extraction method being used was not producing high enough quality, consistent DNA samples. Improvements have already been made and work is still ongoing to optimise both the DNA extraction and the overall system while maintaining a high throughput. An over view of the current inhouse KASP genotyping is given in appendix 2 (section 9.2)

6 General Discussion

6.1 Overview of introgressions produced (collating all three genotyping methods)

The crossing project has successfully produced and genotyped a complete set of addition/substitution lines for the rye accession 390382. Introgressions have been produced containing every chromosome arm of rye but only 1RS, 2RS, 2RL, 3RL, 5RL, 6RL and 7RS have so far been isolated in lines containing no other introgression. Thus, the remaining chromosome arms need further crossing / rounds of self-fertilisation to isolate the introgressions. None of the whole chromosome arm introgressions have been shown to be homozygous, and therefore their position in the wheat genome is not shown in the KASP data. By combining the available GISH data and molecular data it was possible to confirm the presence of seven introgression lines where the introgression is attached to wheat chromatin. Six of these lines contain an introgression equivalent to the size of a chromosome arm and are therefore most probably the product of a translocation, i.e. centric fusions. One introgression from rye chromosome 5L into the D genome is larger and thus likely to be a product of recombination. The rye chromosome arms present in the six lines containing the centric fusions include one line with 2RL, one with 3RS, one with 6S and three lines with 2RS. The remaining rye chromosome arms not described above are also shown to be persistent through each generation and present in many lines in the axiom molecular data therefore it is likely that the majority of rye chromosome arms are present and incorporated within the introgression lines produced, in order to be certain potential lines needs to either be made homozygous and genotyped using KASP or preferentially tested with GISH.

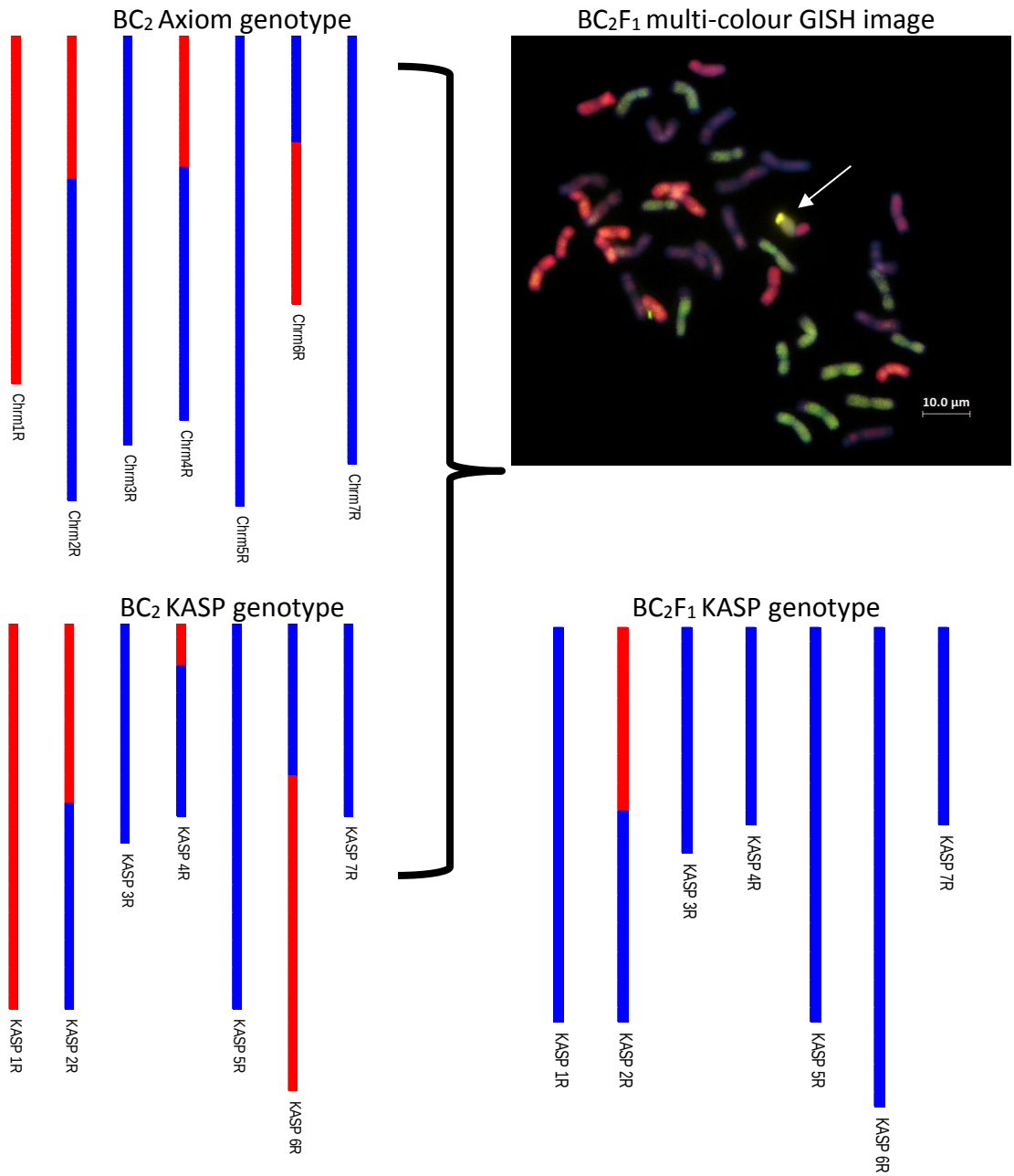

Figure 6.1 The Axiom and KASP genotyping for the same BC_2 introgression line are shown using GGT 2.0 (van Berloo 2008). The KASP genotyping and multi-colour GISH of a BC_2F_1 progeny are also shown. The BC_2F_1 shows a 2RS introgression into the D genome (indicated by the white arrow). Within the GISH image the A genome has been stained green with a *T. urartu* probe. The D genome has been stained red with an *Ae. tauschii* probe. The B genome has not been probed and is shown in blue/purple with a DAPI counterstain. The S. cereale introgression has been stained yellow with an S. cereale probe.

6.2 Efficacy of the project and Improvements

Efficacy of the crossing scheme

The primary goal of this thesis was to produce a range of introgressions that covered the whole rye genome in a wheat background. This target has been achieved, with a range of introgressions covering the whole genome of accessions 390382 and 428373. However, this includes large recombinants, whole chromosome arm translocations and in 428373, some whole chromosome additions. Thus, the size of the introgressions produced were either large or chromosome aberrations that have not been incorporated into the wheat background. This is likely due to a barrier stopping recombination between wheat and rye potential causes for a barrier and methods to overcome it are described below.

The self-fertilisation stage and selection for homozygous lines is still on going as the majority of introgressions are not yet homozygous. Therefore, the lines which will be sent to breeding companies or public breeders and extensively phenotypically investigated have not yet been finalised.

The level of recombination has been shown to vary, however, depending on the species. The level in both *Am. muticum* and *Th. elongatum* was relatively high while rye was extremely low. Work on *Th. bessarabicum* showed a level somewhere in between. Grewal *et al. (2017)* used *Th. bessarabicum* and crossed 1,775 ears producing 10,321 seed (excluding self-seed) and identified 12 different recombination events. In this work, two crossing strategies were employed, both of which exploited the *ph1* mutant. Firstly, *Th. bessarabicum* was crossed as the male parent to *ph1/ph1* mutant wheat, i.e. the same strategy as used in this thesis to produce rye introgression, and secondly, an amphidiploid of *T. turgidum* (Creso *ph1/ph1*) x *Th. bessarabicum* was used as a gene bridge.

The reduced number of recombination events while using comparable sample sizes and techniques therefore implies that the level of recombination between wheat and a wild relative is very much species specific. However, the limitations to recombination are not necessarily the same for the different species. The level of recombination between wheat and rye was particularly low and thus it is possible that there might be more than one barrier and these barriers could be both physical and genetic.

The most likely reason for the lack of recombination between wheat and rye are the differences in the chromosome structures of these two species (described in detail in chapter 5). The differences in structure may be physically stopping homoeologous chromosomes pairing because the chromosomes cannot align properly. One incongruity with this theory is that the chromosomes from group one have the same orthologous gene order in both wheat and rye. Therefore, this theory would predict a higher rate of recombination between the chromosomes in homoeologous group one compared to the other homoeologous groups. Moreover, most of the work in this project utilised rye accession 390382. This accession was proved to be a tetraploid when visualised using GISH. Thus, the F_1 gametes would have contained two rye chromosomes from each linkage group. Hence, these chromosomes would have been able to pair with each other in the F_1 gametes reducing the level of pairing of the rye chromosomes with the homoeologous wheat chromosomes. The tetraploid nature of the accession 390382 may also be the reason it produced the highest number of seed as the rye chromosomes could pair allowing for more normal gametogenesis in the F_1.

It is possible that a genetic barrier such as a *Ph1* homologue may be present within the rye genome preventing homoeologous recombination. To date no such system has been shown to be present in rye (as a mainly diploid species such a system would not be necessary). Multi-colour GISH also showed that recombination had occurred between the wheat genomes

again suggesting that no other system preventing homoeologous recombination was present.

Interestingly the three projects described above all produced more seed per crossed ear as well as more recombinants. The reduced seed and recombinants could both be explained by the rye recombinants being less viable than recombinants produced from other wild relative donors. This may be caused because of a genetic incompatibility between wheat and rye although triticale is a rye/wheat amphidiploid, and this is a very successful hybrid. A second reason is that because of the differences in genome structure between wheat and rye the presence of recombinants produces an unbalanced genome resulting in less seed production and less viable seed Reduced seed production and viability may be due difficulties in endosperm development, which is common in wheat-rye hybrids and therefore more extensive embryo rescue techniques may improve success rates (Brown and Caligari 2008; Molnár-Láng et al. 2015).

Methods to increase recombination

The limited recombination has also affected the other results produced in this work. The low number of recombination events has reduced the resolution of the bin map although the presence of varying sized chromosome aberrations has partially mitigated this. Moreover, the limited amounts of recombination and relatively large introgression sizes means any positive traits are likely to be affected by linkage drag.

There are a few ways the crossing scheme could be altered to attempt to increase recombination rates. One option is to cross rye substitutions lines to a *ph1* mutant. Substitution lines have been used as a bridge to transfer traits from rye into wheat, but the resulting introgressions tend to be large translocations (Jiang et al. 1994b; Lukaszewski 2015). Combining a *ph1* mutant strategy with substitutions is promising for the following reasons. Firstly, the lower proportion of rye chromatin in each cross and the

presence of a near complete wheat genome means seed should be more readily produced. Secondly, it will enable comparisons between each rye chromosome and therefore it may be possible to ascertain if a chromosome or chromosomes are less likely to recombine and thus possibly contain genes that inhibit homoeologous recombination. Finally, the incorporation of a *ph1* mutant may facilitate recombination and therefore produce smaller introgressions than is possible in a method only using substitution lines as a bridge. This approach potentially increases the amount of initial crossing seven-fold (one for each homoeologous group of rye) and the majority of the progeny are likely to lose the rye chromosomes. Therefore, for a crossing scheme using substitution lines to be effective, it would need fast and accurate genotyping to track the rye chromosomes/chromosome segments.

A second and preferred option to improve the crossing scheme is to exploit the *Ph1* supressing loci found in *Am. muticum* (King et al. 2017). The use of *Am. muticum* in a crossing scheme with rye will test if the *Ph1* suppressor in *Am. muticum* is more effective than using *ph1* mutant wheat. The key to adapting this approach is to a produce a wheat plant that contains both the *Am. muticum Ph1* suppressor and rye chromosomes. The most viable option would be to firstly produce a wheat x *Am. muticum* amphidiploid which could then be crossed with rye. The F_1 plants produced that contain the wheat genome, the rye genome and the *Am. muticum Ph1* suppressor should facilitate recombination between wheat and rye chromosomes. Once the F_1 hybrid has been produced, the crossing scheme and genotyping would follow a similar method as described within this thesis.

As well as the substantial changes in approach described above, there are some more subtle changes that may produce more recombinants. The most basic option is to scale up the size of the project, i.e. increase the number of initial crosses. Although this will not lead to an improvement in either seed production or recombination rates it should result in more recombinants

due to the greater number of crosses. A second approach is to attempt to optimise growth conditions in order to promote recombination. Martín et al. (2017) showed that a low temperature (13^{o}C) during meiotic metaphase I and increased nutrient levels increased crossover formation in wheat-rye hybrids, thereby increasing the level of recombination. Treatment with Hoagland solution due to the Mg^{2+} ion level it contains also showed a significant increase in crossover formation. Therefore magnesium supplementation may be a viable way of improving recombination rates (Rey et al. 2018). Rey et al. (2018) proposed that Mg2+ may effect multiple class I interference proteins in the crossover pathway therefore causing increased numbers of crossovers and recombination but the effect reason for Mg2+ increasing cross over formation is not yet known.

Another possible method of improving recombination rates between rye and wheat is to test a larger range of rye accessions to see if any accessions show a higher recombination rate. The use of different rye accessions has the potential to be effective for a number of reasons, primarily because of the variability present within rye. Firstly, the effectiveness of F_1 production is known to be variable through genetic control (Molnár-Láng 2015) and thus it is possible that a more highly crossable accession will produce more recombinants. Secondly, there is structural variation between rye accessions (Alkhimova et al. 1999; Fradkin et al. 2013), and also demonstrated in section 5 by the structural differences shown between Imperial rye and the accession 390382. If the lack of recombination between wheat and rye is due to the structural differences, a rye accession with a more similar chromosome structure to wheat may facilitate higher recombination between wheat and rye.

6.3 Analysis of methods

Overview of introgression characterisation

The aim of characterising introgression lines, irrespective of method, is firstly to determine the presence of an introgression and secondly to provide information about that introgression. Differing methods have distinct strengths and weakness in both introgression detection and data gathered. The following section will compare cytogenetic (GISH) and the molecular (Axiom SNP array) approaches used in this work.

Comparison of techniques

When detecting introgressions, both GISH and the use of SNPs have distinct advantages. The GISH technique is unambiguous and therefore accurately shows an introgression. However, the technique is limited by resolution (very small introgressions might be missed) and also the throughput making the technique unsuitable for the large populations that are common in commercial breading programs. Using SNP arrays to detect introgressions requires considerably more initial input that GISH. SNP markers need to be designed, requiring prior sequence knowledge, and then tested and validated (it is relatively common for SNP markers not to work as expected i.e. markers fail or produce incorrect information). However, once the initial development has been done SNP markers can be used screen many introgression lines giving a much higher throughput compared to GISH. SNP marker systems can be adapted depending on the genotyping aims. A small marker number allows for cheaper and possibly greater throughput genotyping whereas larger marker numbers can be more informative and can screen with a higher resolution. The Axiom wild relative array used large numbers of markers and was therefore designed to produce a high-resolution detailed map of rye and the wheat/rye introgressions, whereas the KASP system, which is still being developed, uses a smaller number of targeted markers to allow for faster, cheaper genotyping at the cost of resolution.

The type of data gathered from these two types of technique is very different. Single colour GISH can identify the presence of an introgression

and the chromosome number and also provides information as to the physical size of the introgression. Single colour GISH also provides useful information about the types of introgression, as it differentiates between rye chromatin that has been recombined or translocated with wheat and chromatin that is completely rye. Single colour GISH can also differentiate between whole chromosome introgressions and all but the largest introgressed chromosome fragments. Multi-colour GISH can gather the same information provided by single colour GISH as well as determining which wheat genome an introgression has recombined/translocated into. GISH does not provide any information about which homoeologous group any of the chromosomes (both rye and wheat) belong to.

The axiom SNP array provides data about the introgressed rye chromatin but because of wheats polyploidy, and the nonspecific nature of the axiom array SNP markers, the results are not informative about the wheat background and cannot discern zygosity as any data is masked by the homoeolgous sequences. Therefore, the axiom SNP array can approximate the genetic size of an introgression and identify the linkage group it belongs to. The lack of information regarding the wheat background means the axiom SNP array cannot tell if the introgression is a chromosomal aberration, recombinant, or a translocation and cannot discern any information about which wheat chromosome an introgression is in. The KASP system also provides the same information as the axiom array but currently with lower resolution. However, many of the KASP markers have been designed to be wheat chromosome specific and therefore can be used to detect if an introgression is heterozygous or homozygous. Moreover, if a recombinant is homozygous, the wheat chromosome specific KASP markers indicate which wheat genome is involved in recombination through the loss of the wheat marker alleles signal.

The initial molecular genotyping was undertaken with the assumption that the rye lines were diploid, though one accession was shown to be tetraploid,

this caused a few complications. Firstly, when using a tetraploid, it is possible two introgressions could occur in the same rye homologous group, in this case a large introgression such as a whole chromosome would mask the presence of any smaller introgressions of the same homologous group. For example, when using the molecular markers, it would be impossible to distinguish an introgression line that only contains a whole 1R introgression from a introgression line containing both a whole 1R introgression and a smaller 1RS introgression. Secondly in the molecular genotyping cannot distinguish between a single introgressed chromosome and multiple chromosomes from the same linkage group (which is only possible when a polyploid wild relative is used to produce the F_1 interspecific hybrid).

Conclusions and potential applications of the GISH and genotyping techniques

All the of above techniques carry different strengths and can fill different niches within an introgression pre-breeding project and beyond. In future projects there will be little benefit to using single colour GISH instead of multi-colour GISH, since multi-colour GISH provides all the information and more provided by single colour GISH with a similar level of throughput. GISH fulfils a key role when validating the molecular genotyping, but the technique does not have a high enough throughput to be the only method of selection in a large breeding or pre-breeding project. GISH is best suited to further investigate smaller numbers of lines once a selection has been made by other genotyping methods.

Molecular techniques are the basis of a modern introgression programme although the optimal technique used will depend on the aims of the aims of the programmes as well as the target and donor species. For similar introgression projects using SNPs, if the target species is a polyploid such as wheat, markers should be ideally designed to be genome specific (or at a minimum a proportion of markers) as this enables identification of zygosity and identifies the genome an introgression is recombined into. The number

of markers used should be tailored to the needs of a programme and its stage. In a pre-breeding programme, the focus is on detecting introgressions and determining the introgression size and composition and as such using the largest possible number of markers is advantageous because more markers (if they are distributed properly) provides a higher resolution, resulting in better detecting power and more precise data about the size of the introgression. After an introgression has been detected and isolated it may be beneficial to use a small number of markers that are genome specific and target a particular introgression. These markers will be effective in selecting for the introgression and detecting if the introgression is homozygous and hence stable and may provide a useful tool to pass onto breeding companies that intend to incorporate introgression lines in commercial breeding schemes.

6.4 Implications of changing technologies

The advancement of next generation sequencing technologies and the continued development of crop plant genome assemblies (Bauer et al. 2017; IWGSC 2018) means genotyping by sequencing is likely to become the major method of genotyping and accessing diversity in crop plants in the near future, i.e. the next 5 – 10 years (Chung et al. 2017; Wallace and Mitchell 2017; Burridge et al. 2018), meaning the SNP based platforms described above will less prevalent. However, the marker sequences they employ can be effectively converted for use in a genotyping by sequencing platform (Burridge et al. 2018).

6.5 Future work and uses for the lines produced

Currently only a small number of introgressions have been made homozygous and therefore the next step for this work is to continue the self-fertilization and selection with the newly developed KASP markers. This process is likely to take between six months and one year. Once homozygous introgression lines have been produced, they will be grown and

bulked to have sufficient seed to be sent to be tested by breeding companies and used in academic phenotyping studies.

The large recombinant that has been produced is present in linkage group 5R and covers the whole long arm of the chromosome and a smaller region the short arm. We have also produced an introgression of the complete long arm of chromosome 5R. Thus, if these two lines were crossed, it should be possible to produce a novel recombinant containing a much smaller region of the original large recombinant.

Some rudimentary phenotyping (tiller number, plant height and some basic photosynthetic measurements) has been undertaken while a subsection of lines progressed through the crossing scheme (data shown in appendix 3) but due to the small number of lines, variable conditions, changing genome composition and lack of replication the results can only be used as an indication the variation present within the introgression lines.

The number of potentially beneficial genes present within rye means that the introgression lines will need to be tested for a range of traits. With an infinite budget and unlimited time, the introgression lines produced should be tested for as large a range of traits as possible. More realistically it would be reasonable to test all introgressions for stem stripe and leaf rusts as rust resistance has been shown to be fairy ubiquitous across all rye chromosomes. Moreover, drought tolerance traits have been shown to be present across all seven chromosomes. For other traits it may be more efficient to test a subset of introgressions either based on the results of previous phenotyping studies, see table 1.4 for a more detailed but far from exhaustive overview of the traits found in rye or to cover the rye genome. For example, if screening for aluminium tolerance it would be reasonable to restrict the trials to introgressions from linkage groups three through seven, or to investigate fusarium head blight resistance starting with introgressions from linkage group three, five and seven. An alternative strategy would be

to screen the larger introgressions for as wide a range of traits as possible. If any of these larger introgressions showed a trait of interest it would then be necessary to produce smaller introgressions from the same region and to screen these for the same trait. Table 6.1 shows a list of traits known in rye and the chromosomes (analogous to linkage group for the introgression) that are likely to carry the trait.

Table 6.1 A selection of traits found in Rye and chromosomes the trait is known to be associated with

Trait	**Chromosomes**
Aluminium tolerance	3R, 4R, 5R, 6R, 7R
Drought tolerance/ Resistance	1R, 2R, 3R, 4R, 5R, 6R, 7R
Dwarfing	1R, 5R, 7R
Frost tolerance	4R, 5R, 7R
Fusarium Head Blight resistance	4R, 5R, 7R
Green Bug resistance	1R
Hessian Fly resistance	6R
Improved Yield	1R
Increased dietary fibre	2R, 5R, 6R
Leaf Rust Resistance	1R
Nematode resistance	6R
Powdery Mildew resistance	1R, 2R, 4R
Soil Borne cereal mosaic virus Resistance	5R
Stem Rust Resistance	1R, 2R, 3R
Stripe Rust resistance	1R
Wheat Spindle Streak Mosaic Virus resistance	7R

Conclusions

The initial aim of this project was to produce a range of introgressions covering the whole rye genome from a few rye accessions. Complete coverage has been achieved with the accession 390382 and more limited success has been achieved with accession 428373. However, due to a lack of recombination, the introgressions are much larger than intended. Only one large introgression was definitively identified as a recombinant and a further 14 whole chromosome arm translocations were detected. Hence further research is needed to induce recombination between wheat and rye. The introgressions produced will be of use in studies investigating rye. All lines produced within the Wheat Research Centre, including the lines produced in this project will be made available free of IP as soon as they have been stabilised (made homozygous) and multiplied.

The secondary aim of this work was to develop effective methods of detecting and characterising wheat-rye introgression lines. Multi-colour GISH was shown to effectively identify rye chromatin, the wheat genome the rye chromatin has been introgressed into and was effective in confirming results from SNP markers. SNP markers from the axiom wild relative array were successfully used to produce a bin map of rye, which is effective at identifying and tracking introgressions.

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8 Appendix (1)

1.6% agarose gel (250ml)

Agarose	4g
1xTAE buffer	250ml
Ethidium bromide	10µl

Agarose was added to 1xTAE buffer in a conical flask and heated and mixed until completely dissolved. The conical flask was left to cool for ~3minutes then ethidium bromide added and poured into mould and left to set before use.

5% hypochlorite solution, CINaO

Sigma Alrich 017-001-00-1 10% sodium hypochlorite solution was diluted in a 1:1 ratio with sterile deionised water with 1 drop of tween 20 added to produce 5% CINaO.

Hortifeed nutrient solution

500g of Hortifeed Standard (http://www.hortifeeds.co.uk) was diluted into for 5l of water as a stock solution then further diluted by 1:100 in the glass house.

Sucrose Media (1l)

Murashige and Skoog dried medium 0.441%	4.41g
Sucrose 3%	30g
Agar 0.8%	8g

Dissolved in purified autoclaved water up to 1l then adjusted to pH 5.8 with 10M sodium hydroxide and 1.0M hydrochloric acid.

Glasshouse conditions

The glass houses operated with 16 hours of light at 20°C and 8 hours of dark at 15°C.

DNA Extraction buffer (200ml)

0.1M Tris-HCL (pH 7.5)	20 ml
0.05M EDTA	20 ml
10% SDS	25 ml
Sterile Deionized water	135 ml

Salmon Sperm DNA working solution (140µg/ml) (50ml)

50X TAE (pH 6.3)	5ml
Salmon Sperm DNA (10mg/ml)	0.7ml
Water	44.3ml

Stored at 4°C in a fridge

3M sodium acetate pH 5.2 (NaOac) (100 ml)

Sodium acetate (m.w.-136.08)	40.8g
Sterile Deionized water	70ml
Adjust pH to 5.2	
Sterile Deionized water	upto 100ml

Sterilise the solution by 0.22 µm membrane filter

Precipitation Solution (100 ml)

A 9:1 ratio of 100% ethanol: 3M sodium acetate pH 5.2

100% ethanol	90 ml
3M sodium acetate pH 5.2	10 ml

20x SSC (1l)

NaCl	175.3g
Sodium citrate	88.2g
Adjusted to	pH 7.0

Water	upto 1l

2x SSC (for 1l)

Diluted from 20x SSC

20x SSC	100mls
Water	900mls

10 x .TE

100mM Tris

10mM EDTA

Adjusted to pH7.5

1 x TE (100ml)

Diluted from 10 x TE

10xTE	10ml
Water	90ml

2x SSC+1x T.E. (10ml)

1:1 ratio of 2x SSC and 1x TE

2x SSC	5ml
1x TE	5ml

Vectashield mounting medium with DAPI (20ml)

1:3 ratio of vectashield mounting medium with DAPI (vector laboratories inc Burlingame, CA) and 1 X TE

Vecatshield	5ml
1 x TE	15ml

9 Appendix (2) supplementary work

9.1 Preliminary FISH work

Work producing a FISH karyotype for the accession line 390382 is still undergoing as until recently (Less than one month prior to publication) a complete set of addition/substitution lines from the accession 390382 was not available. The addition lines have now been produced as part of this thesis and therefore the production of a karyotype is now possible. Below is the methodology that could be used.

Background

FISH can be used to produce a karyotype. The repetitive sequences pAs1, pSc119.2, and pTa71 are some of the most commonly used FISH probes and can characterise wheat, rye, and wheat-rye hybrids (Bedbrook et al. 1980; McIntyre et al. 1990; Nagaki et al. 1995; Cuadrado et al. 1997; Contento et al. 2005; Fujisawa et al. 2006; Fu et al. 2013; Hao et al. 2013). Fradkin et al. (2013) used FISH probes, pSc74, pSc250, and pSc200, which bind to rye but does not bind to wheat, to identify and then differentiate rye varieties within triticale. Discrimination between rye accessions is possible because rye has a more variable genome than wheat because Rye is allogamous. These changes in genome can be exploited to differentiate rye but varieties but make developing introgressions from novel rye varieties difficult because an existing karyotype is unlikely.

FISH karyotypes have been effectively used to identify rye chromosomes in triticale (Fradkin et al. 2013; Li et al. 2015a). FISH karyotypes differ depending on the relative positions of the repetitive sequence used.

FISH Karyotyping (method)

The repetitive sequences pSc200 and pSc250 were chosen as they specifically bind to rye along with and used alongside pS119.2, pASII which are less specific repetitive sequence to produce the karyotype the wheat chromosomes.

Producing Template DNA for FISH

The primers used for pSc200 were; primer 1: 5'-GAGTCTCGATCAATTTCGG-3' and primer 2: 5'-GCAAGTGAG GAGACAAGC-3'; and the primers for pSc250 used were; primer 1: 5'-GTTCGAAAATAATGG GCC-3' and primer 2: 5'-CCAACCACTAAATCATTCG-3'. PCR was performed with rye total genomic DNA, extracted as above, using the following conditions: 5 min at 94 °C; followed by 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 90 s at 72 °C; with a final extension of 5min at 72 °C (Vershinin 1995; Fradkin et al. 2013)

Table 9.1. *The solution prepared to stain 1 slide with FISH. The wave probes excitation wave length (nm) is given in bold along with its nearest colour*

Probe	***Volume***
pS119.2 ***GREEN 488***	*1 µl*
pASII ***RED 594***	*1.5 µl*
pSc200 **GOLD 546**	1 µl
2xSSC 1xTE to total 10 µl	

pS119.2 and pASII probes were produced from plasmids containing the repetitive described in 2.2. pS119.2 was fluorescently labelled with, 5-dUTP ChromaTide(r) Alexa Fluor(r) 488 nm (www.thermofisher.com), green pASII was fluorescently labelled with, 5-dUTP ChromaTide(r) Alexa Fluor(r) 594nm (www.thermofisher.com), Red. The pSC250 PCR was unsuccessful so was not used for probe production, The pSc200 PCR product was used to fluorescently labelled with 5-dUTP ChromaTide(r) Alexa Fluor(r) 546 nm

(www.thermofisher.com), Gold. Probes were produced as described in section 2.2 page 36-38 Each slide was stained with the mixture shown in table 9.1. Slides were probed as described in section 4.2.

Karyotyping (yet to be completed)

Substitution or addition lines for every chromosome of the rye accession line 390382 were produced as a by-product of the crossing scheme described in chapter three and identified using the molecular genotyping described in chapter five. These lines only become available one month prior to submissions and therefore the karyotype has not been produced. To produce a Karyotype each line will be analysed with the FISH probes described above then the data will be used to produce a karyotype to analyse future introgression lines.

9.2 In house KASP

A subsection 42 of markers, designed as described in section 5.6, chosen to have one marker per chromosome arm of wheat was tested on DNA was extracted using a proprietary single step DNA extraction method, that from 89 experimental introgression lines produced in chapter 3 and three paragon wheat controls three rye, accession 390382 controls. Primers for each marker were produced by LGC (http://www.lgcgenomics.com), the 1µl of each allele specific forward primer and the reverse primer were diluted to x 74 using sterile deionised water.

5 µl of template DNA from each sample or control was aliquoted into 384 well PCR plates and dried at 80°C for 15 minutes. A reaction mixture of 2.43µl dH_20, 2.43µl KASP V4.0 2X Master mix (KBS-1016-003 http://www.lgcgenomics.com) and 0.068µl of the x 74 primer mixture was added to each well. PCR was then preformed using the following protocol: A initial hold step at 94°C for 10 minutes. Secondly 10 cycles of 94°C for 15 seconds then, 65°C for one minute. The third stage was 50 cycles of 94°C for 15 seconds then 57°C for 1 minute and finally a holding stage of 4°C once the PCR has completed.After PCR was completed fluorescence was measured using a QuantStudio™5 system (http://www.thermofisher.com) and scored using the QuantStudio™ Design and Analysis Software v1.4.3 (http://www.thermofisher.com).

Of the 42 markers tested 12 worked as expected with the controls segregating properly, and therefore meaningful analysis of the genotypes was not possible. The markers used, and the experimental lines had been previously tested by LGC as described in chapter 5. Therefore, the results were due to procedural error. Therefore, the process is currently being optimised by the University of Nottingham Wheat Research Center and some success has been achieved by optimising the DNA extraction method

using wild relatives over than rye. Currently the optimised DNA extraction has not been tested on rye.

10 Appendix (3) Supplementary Data

List of lines genotyped with the Axoin Wild relative array.

BC1-146	BC2-311A	BC3-137A	BC3F1-158B
BC1-153	BC2-311B	BC3-137B	BC3F1-158C
BC1-154	BC2-311C	BC3-138A	BC3F1-159A
BC1-155	BC2-311D	BC3-139A	BC3F1-161A
BC1-156	BC2-311E	BC3-140A	BC3F1-161B
BC1-157	BC2-311F	BC3-140B	BC3F1-161C
BC1-170A	BC2-311G	BC3-141A	BC3F1-161D
BC1-170B	BC2-314A	BC3-224A-	BC3F1-161E
BC1-170C	BC2-315A	BC3-226A-	BC3F1-162A
BC1-170D	BC2-316A	BC3-229A-	BC3F1-162B
BC1-171A	BC2-317A	BC3-229B-	BC3F1-162C
BC1-171B	BC2-318A	BC3-230A-	BC3F1-162D
BC1-203	BC2-319A	BC3-231A-	BC3F1-162E
BC1-268B	BC2-320A	BC3-232A-	BC4-152A
BC1-374A	BC2-321A	BC3-232B-	BC4-152B
BC1-374B	BC2-324A	BC3-351A	BC4-152C
BC1-92B	BC2-555A	BC3-351B	BC4-152D
BC1-94	BC2-556A	BC3-351D	BC4-152E
BC1-95	BC2-557	BC3-352A	BC4-155A
BC1-96	BC2-559	BC3-352B	BC4-155B
BC1-97	BC2-561	BC3-352C	BC4-155C
BC1-98	BC2-562	BC3-352D	BC4-155D
BC2-190	BC2-564	BC3-353B	BC4-156A
BC2-191	BC2-565A	BC3-353C	BC4-156B
BC2-289A	BC2-565B	BC3-354A	BC4-156C
BC2-290A	BC2-655A	BC3-355A	BC4-156D
BC2-292A	BC2-655B	BC3-355B	BC4-156E
BC2-293A	BC2-656A	BC3-355C	BC4-157A
BC2-296A	BC2-658A	BC3-355D	BC4-157B
BC2-298A	BC2-659A	BC3-356A	BC4-157C
BC2-299A	BC2-660A	BC3-356C	BC4-157D
BC2-300A	BC2-660B	BC3-359A	BC4-158A
BC2-301A	BC2-661A	BC3F1-156A	
BC2-302A	BC2-662A	BC3F1-156B	
BC2-303A	BC2-666A	BC3F1-156C	
BC2-304A	BC2-666B	BC3F1-157A	
BC2-305A	BC2-667A	BC3F1-157B	
BC2-306A	BC2-667B	BC3F1-157C	
BC2-307A	BC2-696A	BC3F1-157D	
BC2-309A	BC3-136A	BC3F1-157E	
BC2-310A	BC3-136B	BC3F1-158A	

List of lines genotyped using the KASP system by LGC

Rye 390382	BC2F1_72Q	BC2_306F	BC3F1_92A
Rye 428373	BC2F1_72R	BC2_565C	BC3F1_92B
Chinese Spring	BC2F1_72S	BC2_728A	BC3F1_92C
Highbury	BC2F1_72T	BC2_730A	BC3F1_92D
Paragon	BC2F1_73 O	BC2_731A	BC3F1_92E
Pavon	BC2F1_73A	BC2_732A	BC3F1_92F
Add-1R	BC2F1_73B	BC2_733A	BC3F1_92G
Add-2RS	BC2F1_73C	BC2_733B	BC3F1_92H
Add-3R	BC2F1_73D	BC2_734A	BC3F1_92i
Add-4R	BC2F1_73E	BC2_734C	BC3F1_92J
Add-5R	BC2F1_73F	BC2_736A	BC3F1_93A
Add-5RL	BC2F1_73G	BC2_736B	BC3F1_93B
Add-6R	BC2F1_73H	BC2_736C	BC3F1_93C
Add-6RL	BC2F1_73i	BC2_738A	BC3F1_93D
BC1-171A	BC2F1_73J	BC2_738B	BC3F1_93E
BC2-190	BC2F1_73K	BC2_739A	BC3F1_93F
BC2-289A	BC2F1_73L	BC2_739B	BC3F1_93G
BC2-298A	BC2F1_73M	BC2_739C	BC3F1_93i
BC2-309A	BC2F1_73N	BC2_739D	BC3F1_93J
BC2-318A	BC2F1_73P	BC3-139A	BC3F1_94A
BC2-556A	BC2F1_74C	BC3-227A	BC3F1_94B
BC2-565A	BC2F1_74D	BC3-229A-	BC3F1_94C
BC2-565B	BC2F1_74E	BC3-232A	BC3F1_94D
BC2-656A	BC2F1_74G	BC3-319A	BC3F1_94E
BC2-659A	BC2F1_74i	BC3-352A	BC3F1_94F
BC2-660A	BC2F1_74J	BC3-352B	BC3F1_95A
BC2-671A	BC2F1_75A	BC3-352C	BC3F1_95B
BC2-F1-254G	BC2F1_75B	BC3-353B	BC3F1_95C
BC2-F1-255D	BC2F1_75C	BC3-354A	BC3F1_95D
BC2F1-255B	BC2F1_75D	BC3-644A	BC3F1_95E
BC2F1_191A	BC2F1_75E	BC3-644C	BC3F1_95F
BC2F1_191B	BC2F1_75F	BC3F1_101A	BC3F1_95G
BC2F1_191C	BC2F1_75G	BC3F1_101B	BC3F1_95H
BC2F1_191D	BC2F1_75H	BC3F1_101C	BC3F1_95i
BC2F1_191E	BC2F1_75i	BC3F1_101D	BC3F1_95J
BC2F1_191F	BC2F1_75J	BC3F1_101E	BC3F1_96A
BC2F1_191G	BC2F1_76A	BC3F1_101F	BC3F1_96B
BC2F1_191H	BC2F1_76B	BC3F1_101G	BC3F1_96C
BC2F1_191i	BC2F1_76C	BC3F1_101H	BC3F1_96D
BC2F1_191J	BC2F1_76D	BC3F1_101i	BC3F1_96E
BC2F1_191K	BC2F1_76E	BC3F1_101J	BC3F1_96F
BC2F1_192A	BC2F1_76F	BC3F1_102A	BC3F1_96G
BC2F1_192B	BC2F1_78 O	BC3F1_102B	BC3F1_96H

BC2F1_192C	BC2F1_78A	BC3F1_102C	BC3F1_96J
BC2F1_192D	BC2F1_78B	BC3F1_102D	BC3F1_97A
BC2F1_192E	BC2F1_78C	BC3F1_102E	BC3F1_97B
BC2F1_192F	BC2F1_78D	BC3F1_102F	BC3F1_97C
BC2F1_192G	BC2F1_78E	BC3F1_102G	BC3F1_97D
BC2F1_192H	BC2F1_78F	BC3F1_102H	BC3F1_97E
BC2F1_192i	BC2F1_78G	BC3F1_102i	BC3F1_97F
BC2F1_193A	BC2F1_78K	BC3F1_102J	BC3F1_97G
BC2F1_253A	BC2F1_78L	BC3F1_103A	BC3F1_97H
BC2F1_254A	BC2F1_78M	BC3F1_103B	BC3F1_97i
BC2F1_254B	BC2F1_78N	BC3F1_103C	BC3F1_97J
BC2F1_254C	BC2F1_78P	BC3F1_103D	BC3F1_98A
BC2F1_254D	BC2F1_78Q	BC3F1_104A	BC3F1_98B
BC2F1_254E	BC2F2_38A	BC3F1_105A	BC3F1_98C
BC2F1_254F	BC2F2_38B	BC3F1_180B	BC3F1_99A
BC2F1_255A	BC2F2_38C	BC3F1_181A	BC3F1_99B
BC2F1_255C	BC2F2_38D	BC3F1_181B	BC3_351E
BC2F1_257A	BC2F2_38F	BC3F1_181C	BC3_351F
BC2F1_27A	BC2F2_38G	BC3F1_182A	BC3_351G
BC2F1_27B	BC2F2_39A	BC3F1_182B	BC3_351H
BC2F1_27C	BC2F2_39B	BC3F1_182C	BC3_635A
BC2F1_27D	BC2F2_39C	BC3F1_182D	BC3_636A
BC2F1_28C	BC2F2_39D	BC3F1_85A	BC3_636C
BC2F1_29A	BC2F2_39E	BC3F1_85B	BC3_636D
BC2F1_29B	BC2F2_39F	BC3F1_85C	BC3_636E
BC2F1_29C	BC2F2_39G	BC3F1_85D	BC3_637A
BC2F1_29D	BC2F2_39H	BC3F1_85E	BC3_637B
BC2F1_29E	BC2F2_40A	BC3F1_85F	BC3_637C
BC2F1_29F	BC2F2_40B	BC3F1_85G	BC3_637E
BC2F1_30A	BC2F2_40C	BC3F1_85H	BC3_638A
BC2F1_30B	BC2F2_40D	BC3F1_85i	BC3_638B
BC2F1_30C	BC2F2_40E	BC3F1_85J	BC3_638C
BC2F1_30D	BC2F2_40F	BC3F1_86A	BC3_638D
BC2F1_30E	BC2F2_40G	BC3F1_86B	BC3_638E
BC2F1_30G	BC2F2_40H	BC3F1_86C	BC3_639A
BC2F1_30H	BC2F2_40i	BC3F1_86D	BC3_639B
BC2F1_65A	BC2F2_41A	BC3F1_86E	BC3_640A
BC2F1_65B	BC2F2_41B	BC3F1_86F	BC3_640B
BC2F1_65C	BC2F2_41C	BC3F1_86G	BC3_640C
BC2F1_65D	BC2F2_41D	BC3F1_86H	BC3_640D
BC2F1_65E	BC2F2_41E	BC3F1_86i	BC3_640E
BC2F1_65F	BC2F2_41F	BC3F1_86J	BC3_640F
BC2F1_65G	BC2F2_41G	BC3F1_87A	BC3_640G
BC2F1_65H	BC2F2_41H	BC3F1_87B	BC3_643A
BC2F1_65i	BC2F2_41i	BC3F1_87C	BC3_644A

BC2F1_65J	BC2F2_42A	BC3F1_88A	BC3_644B
BC2F1_65K	BC2F2_42B	BC3F1_88B	BC3_644C
BC2F1_65L	BC2F2_42C	BC3F1_88C	BC3_645A
BC2F1_66A	BC2F2_42D	BC3F1_88D	BC3_645B
BC2F1_66B	BC2F2_42E	BC3F1_89A	BC3_645C
BC2F1_66C	BC2F2_42F	BC3F1_89B	BC3_648A
BC2F1_67A	BC2F2_42G	BC3F1_89C	BC3_648B
BC2F1_68A	BC2F2_42H	BC3F1_89D	BC3_648C
BC2F1_68B	BC2F2_42i	BC3F1_89E	BC3_648D
BC2F1_68C	BC2F2_42J	BC3F1_89F	BC3_649A
BC2F1_68D	BC2F2_43A	BC3F1_89G	BC3_649B
BC2F1_69A	BC2F2_43B	BC3F1_89H	BC3_649C
BC2F1_70A	BC2F2_43C	BC3F1_89i	BC3_657A
BC2F1_70B	BC2F2_43D	BC3F1_90A	BC3_658A
BC2F1_71A	BC2F2_43E	BC3F1_90B	BC3_658B
BC2F1_71B	BC2F2_43F	BC3F1_90C	BC3_659A
BC2F1_71C	BC2F2_43G	BC3F1_90D	BC3_659C
BC2F1_71D	BC2F2_43H	BC3F1_90E	BC3_659D
BC2F1_72 O	BC2F2_43J	BC3F1_90F	BC3_661A
BC2F1_72A	BC2F2_44A	BC3F1_90G	BC3_662A
BC2F1_72B	BC2F2_44B	BC3F1_90H	BC3_662B
BC2F1_72C	BC2F2_44C	BC3F1_90i	BC4_228A
BC2F1_72D	BC2F2_44D	BC3F1_90J	BC4_228B
BC2F1_72E	BC2F2_44E	BC3F1_91A	
BC2F1_72F	BC2F2_44F	BC3F1_91B	
BC2F1_72G	BC2F2_44G	BC3F1_91C	
BC2F1_72J	BC2F2_44H	BC3F1_91D	
BC2F1_72K	BC2F2_44i	BC3F1_91E	
BC2F1_72L	BC2_306B	BC3F1_91F	
BC2F1_72M	BC2_306C	BC3F1_91G	
BC2F1_72N	BC2_306D	BC3F1_91H	
BC2F1_72P	BC2_306E	BC3F1_91i	

List of line used in House for KASP

BC2-306D	BC3-351F	BC3-F2-88O
BC2-306E	BC3-351G	BC4-228A
BC2-730A	BC3-351H	BC4-228B
BC2-732A	BC3-634A	BC4-F1-114G
BC2-733A	BC3-635D	BC4-F1-120A
BC2-734A	BC3-636C	BC4-F1-120B
BC2-738D	BC3-636E	BC4-F1-120C
BC2-739D	BC3-637A	Paragon_1
BC2-F1-254A	BC3-637B	Paragon_2
BC2-F1-254B	BC3-637E	Paragon_3
BC2-F1-254D	BC3-638A	Rye_390382_1
BC2-F1-254F	BC3-638B	Rye_390382_2
BC2-F1-254G	BC3-638C	Rye_390382_3
BC2-F1-255C	BC3-638D	
BC2-F1-265A	BC3-638E	
BC2-F1-266B	BC3-645A	
BC2-F1-266C	BC3-648A	
BC2-F1-266E	BC3-648C	
BC2-F1-266F	BC3-659C	
BC2-F1-266G	BC3-659D	
BC2-F1-266H	BC3-662A	
BC2-F1-266I	BC3-662B	
BC2-F1-266J	BC3-705A	
BC2-F1-266K	BC3-F1-181C	
BC2-F1-68C	BC3-F1-182A	
BC2-F2-684A	BC3-F1-182C	
BC2-F2-684B	BC3-F1-182D	
BC2-F2-684C	BC3-F1-252F	
BC2-F2-684D	BC3-F1-252G	
BC2-F2-684E	BC3-F1-252I	
BC2-F2-684F	BC3-F1-255A	
BC2-F2-684G	BC3-F2-87A	
BC2-F2-685B	BC3-F2-87G	
BC2-F2-685C	BC3-F2-87H	
BC2-F2-685F	BC3-F2-87I	
BC2-F3-12B	BC3-F2-87K	
BC2-F3-13A	BC3-F2-88C	
BC2-F3-13C	BC3-F2-88G	
BC2-F3-14A	BC3-F2-88K	
BC2-F3-14B	BC3-F2-88L	
BC2-F3-14C	BC3-F2-88M	

Linkage group one rye bin map blast data

The markers are ordered according to the rye bin map and the IWGSC reference position is shown the chromosome with the highest blast result has been marked in green

Marker Name	IWGSC position	Chrm	IWGSC position	Chrm	IWGSC position	Chrm
AX-94627714	9851176	1A	11699627	1B	8626458	1D
AX-94923857	11691168	1A	15142424	1B		
AX-94590720	22204866	1A	16749452	1B	11498369	1D
AX-95241582	19694408	1A	27702045	1B	18068035	1D
AX-94977369	28219947	1A	43366775	1B	27693629	1D
AX-94948332	28784344	1A	45649568	1B	28202884	1D
AX-94635206	35526089	1A	56172535	1B	36534708	1D
AX-95163118	36162529	1A	56357926	1B	37221045	1D
AX-94565423	48691702	1A	69605176	1B	49588221	1D
AX-94600202	48692194	1A	69605659	1B	49588712	1D
AX-94559449	67967070	1A	110751408	1B	69179557	1D
AX-94428885	72380154	1A	115009642	1B	73144842	1D
AX-94618682	72380213	1A	689237110	1B	73144901	1D
AX-94598986	593696898	1A	115684570	1B	73679636	1D
AX-94838542	98610732	1A	132271340	1B	80447489	1D
AX-94668518	82120828	1A	139389795	1B	86924245	1D
AX-94784779	155289523	1A	689237110	1B	137437734	1D
AX-94882839			107235861	1B		
AX-94917835	239909239	1A	267976758	1B	193508352	1D
AX-94991357	256422877	1A	296073920	1B	202570195	1D
AX-94549470			332290382	1B		
AX-94477543	368069254	1A	368069254	1B	233906908	1D
AX-94762019	309849885	1A	340675774	1B	235673389	1D
AX-94456521	319818622	1A	363932007	1B	250140327	1D
AX-94416910	321471109	1A	362119137	1B	251706671	1D
AX-94783071	324645163	1A	358710169	1B	253217531	1D
AX-94700816	376886974	1A	408741455	1B	302378861	1D
AX-94815097	376676515	1A	409115644	1B	302708755	1D
AX-94509407			409127696	1B	302822955	1D
AX-94722723	387115603	1A	416905104	1B	308266123	1D
AX-94627870	387117850	1A	416907767	1B	308268828	1D
AX-95215437	408083406	1A	441526965	1B	495453186	1D
AX-94828230	400551252	1A	433888850	1B		
AX-94820157	420105709	1A	436154834	1B	324418511	1D
AX-95241585	406709461	1A	441567900	1B	327991942	1D
AX-94446364	427413543	1A	444899433	1B	330550360	1D

AX-94799746	432366172	1A	450542432	1B	335272744	1D
AX-94496605	440395861	1A	456304540	1B	341175832	1D
AX-94734544	447223191	1A	466991686	1B	347204338	1D
AX-94936271	448519585	1A	469421810	1B	348061482	1D
AX-94482529	451640543	1A	473298965	1B	351256775	1D
AX-94856060	593696898	1A	473879090	1B	352194358	1D
AX-95130810	460952185	1A	483797680	1B	361908155	1D
AX-94564167	464432024	1A	488677457	1B	365727983	1D
AX-94644793	476613986	1A	502952636	1B	376783029	1D
AX-94737787	482356678	1A	511858898	1B	382635720	1D
AX-94408077	485937712	1A	518247069	1B	386434123	1D
AX-94616595	499045483	1A	542618869	1B	403732177	1D
AX-95165383	502274271	1A	546719268	1B	406974719	1D
AX-94637738	505876977	1A	552453235	1B	410464703	1D
AX-94777625	517010379	1A	569679627	1B	421037416	1D
AX-94927609	517614136	1A	570463526	1B	422056065	1D
AX-94849593	530831973	1A	583559001	1B	432795724	1D
AX-94463413	536272446	1A	669910114	1B	441207321	1D
AX-95199064	539561033	1A	603710539	1B	444336901	1D
AX-95239366	539749370	1A	604411040	1B	444865694	1D
AX-94795738	543644981	1A	612814794	1B	448013585	1D
AX-94873049	545125773	1A	617224296	1B	450227591	1D
AX-94382911	560930651	1A	646634917	1B	468467843	1D
AX-95139021	576208602	1A	668040950	1B	480346259	1D
AX-94634352	579422797	1A	673129494	1B	483514244	1D
AX-94601839	579422618	1A	673129673	1B	483514423	1D
AX-94608604	580335301	1A	673256217	1B	483879638	1D

Linkage group two rye bin map blast data

The markers are ordered according to the rye bin map and the IWGSC reference position is shown the chromosome with the highest blast result has been marked in green

Marker Name	IWGSC position	Chrm	IWGSC position	Chrm	IWGSC position	Chrm
AX-94634113	53787823	2A	81761117	2B	52232469	2D
AX-94856223	55173586	2A	85321411	2B	54673755	2D
AX-94956253	59323582	2A	91836874	2B	59007490	2D
AX-94799451	58672342	2A	92797765	2B	59551292	2D
AX-94663017	62369345	2A	97394507	2B	62031868	2D
AX-94924010	63147378	2A	97935847	2B	62296601	2D
AX-94606624			98254916	2B	62551547	2D
AX-94717113	78329856	2A	121370067	2B	78391609	2D
AX-94643588	78844055	2A	121676875	2B	78562287	2D
AX-94661962	79163499	2A	121991121	2B	78692392	2D
AX-94484294	79205226	2A	122696953	2B	78764498	2D
AX-94876219	80618206	2A	131584591	2B	80037502	2D
AX-95009765	86441953	2A	139074239	2B	86082320	2D
AX-94531232	102327462	2A	154720797	2B	103166881	2D
AX-95113524	102686603	2A	154986086	2B	106321792	2D
AX-95013802	115414000	2A	164419618	2B	113912198	2D
AX-95090395	116000004	2A	43616008	2B	114547371	2D
AX-94985650	118672527	2A	167799471	2B	117228514	2D
AX-94920067	122008744	2A	171997673	2B	119662126	2D
AX-94926150	125130612	2A	174345511	2B	120973568	2D
AX-94911592	126996226	2A	175459459	2B		
AX-94940867	126995963	2A	175459196	2B		
AX-94575652	127154703	2A	175537882	2B	122703256	2D
AX-95176241	159937979	2A	199123438	2B	141191516	2D
AX-94703888	167358284	2A	214837594	2B	157396200	2D
AX-94729799	171078836	2A			159851453	2D
AX-94535299	178465825	2A	225820167	2B		
AX-94868570	207588930	2A	249517808	2B	193393841	2D
AX-95217835	277602482	2A	259782508	2B	205619302	2D
AX-94864501	324615892	2A	331012099	2B	218046021	2D
AX-95245005	240760450	2A	345763851	2B	222648159	2D
AX-95149610	240760476	2A	345763894	2B	222648202	2D
AX-94639026	289759661	2A	298151426	2B	249195239	2D
AX-94666301	244315809	2A	305328372	2B	261733556	2D
AX-94561243	366459335	2A	371754475	2B	293656139	2D
AX-94628262	658744713	2A	606484170	2B	514657427	2D
AX-94498626	293152448	2A	285054774	2B	257388324	2D

AX-94512204	384347860	2A	367468257	2B	305522664	2D
AX-95083240	394021452	2A	393464538	2B	324594579	2D
AX-95166376	437976167	2A	399353904	2B	330751699	2D
AX-94533178	445999125	2A	403557876	2B	335411245	2D
AX-95097228	470432799	2A	418778273	2B	352626737	2D
AX-94591112	471005113	2A	419321891	2B	352904818	2D
AX-94711036	473409294	2A	421084226	2B	354321456	2D
AX-94994927	475459741	2A	423664965	2B	355683304	2D
AX-94902345	485764072	2A	429253595	2B	360306568	2D
AX-94745279	497441725	2A	436028762	2B	367229112	2D
AX-94725279	503004341	2A	441861287	2B	372274192	2D
AX-95228642	504071442	2A	442307261	2B	372695560	2D
AX-94432991	504076165	2A	442310203	2B	372700255	2D
AX-94567305	504273469	2A	442797642	2B	373227516	2D
AX-94628644	509076212	2A	447746156	2B	376324354	2D
AX-94677144	512228725	2A	449168445	2B	378056982	2D
AX-95090932	518336853	2A	454019108	2B	383560478	2D
AX-94803709	527458096	2A	461978835	2B	390730561	2D
AX-94775107	527920308	2A	462893688	2B	391279088	2D
AX-94899058	543620213	2A	481048771	2B	405415989	2D
AX-95226649	562460924	2A	492958059	2B	420525583	2D
AX-94544799	577306466	2A	510589844	2B	431678654	2D
AX-94447470	585662171	2A	521332863	2B	441770320	2D
AX-94826072	589692671	2A	525522142	2B	446260341	2D
AX-94680402	593502125	2A	529108148	2B	449512002	2D
AX-94405124	602810597	2A	541107999	2B	459853043	2D
AX-94522494	605800945	2A	543191083	2B	461302469	2D
AX-95086997	606676643	2A	543707044	2B	461546258	2D
AX-94965918	606677767	2A	543708169	2B	461547383	2D
AX-94502406	608033039	2A	546587465	2B	468099947	2D
AX-94848399	611953992	2A	549191566	2B	470224167	2D
AX-94949353	615290234	2A	553574116	2B	473168628	2D
AX-94973623	615359828	2A	553650313	2B	473291373	2D
AX-94996857	617655328	2A	556202596	2B	475093634	2D
AX-94620731	619202741	2A	557992838	2B	476837970	2D
AX-94676788	619757897	2A	559309457	2B	477273246	2D
AX-94736800	622456270	2A	562977345	2B	480131045	2D
AX-94758297	641953290	2A	583674889	2B	497362554	2D
AX-94678904	644084941	2A	587169771	2B	499155258	2D
AX-94935291	654914415	2A	600746970	2B	511694991	2D
AX-94488606	659279305	2A	607056709	2B	515582380	2D
AX-95141943	667657399	2A	615365514	2B	522544394	2D
AX-94557958	668070498	2A	616497257	2B	522993230	2D
AX-94768754	676715373	2A	632010618	2B	532177330	2D
AX-95008135	678671624	2A	636776066	2B	535571994	2D

AX-94410030	678672009	2A	636776453	2B	535572376	2D
AX-94644873	680097012	2A	640490061	2B	537704761	2D
AX-95222889	683013811	2A	643684994	2B	539429254	2D
AX-94584055	685036957	2A	647109210	2B	542150430	2D
AX-94557836	699514935	2A	667417304	2B	558803643	2D
AX-94567662	704758812	2A	675337578	2B	564077736	2D
AX-94478396	707749628	2A	680768416	2B	568230880	2D
AX-95102643	719479322	2A	706296149	2B	585389042	2D
AX-95123223			710988127	2B		
AX-94592148	743387977	2A	746037456	2B	611160654	2D
AX-94818511	753705005	2A	763878535	2B	621977878	2D
AX-94613960	753705202	2A	763878732	2B	621978075	2D
AX-94829158	765702806	2A	800333283	2B	640208560	2D
AX-95084761			784545648	2B	648468489	2D

Linkage group three rye bin map blast data

The markers are ordered according to the rye bin map and the IWGSC reference position is shown the chromosome with the highest blast result has been marked in green

Marker Name	IWGSC position	Chrm	IWGSC position	Chrm	IWGSC position	Chrm
AX-94785800	24056480	3A	17552227	3B	13145278	3D
AX-94891646	31659896	3A	37592777	3B	22047410	3D
AX-94494434	35763720	3A	42005610	3B	26035474	3D
AX-94620080	50382889	3A	60624065	3B	39032595	3D
AX-94536318	61348855	3A	77114279	3B	48867078	3D
AX-94475339	68287124	3A	93791182	3B	59296610	3D
AX-94686583	70705879	3A	97752748	3B	61680865	3D
AX-94629757	75413556	3A	106301942	3B	64649108	3D
AX-94402778	88857098	3A	120213402	3B	75354696	3D
AX-95098138	97051730	3A	127201906	3B	81826415	3D
AX-94468885	102273250	3A	133255193	3B	85250955	3D
AX-94837190	112919238	3A	168058080	3B	116008824	3D
AX-95076032	130836247	3A	174389958	3B	123292905	3D
AX-94745249	131416134	3A	175268126	3B	123512947	3D
AX-94650966	142578013	3A	185784791	3B	130500033	3D
AX-94999625	144165408	3A	186450141	3B	131430001	3D
AX-94562576	191145804	3A	238898155	3B	164058762	3D
AX-94557609	209406657	3A	249093066	3B	172020829	3D
AX-94647370	214291093	3A	252523432	3B	174923555	3D
AX-94601065	214291151	3A	252523490	3B	174923613	3D
AX-94579237	220887779	3A	255664242	3B	178588787	3D
AX-94664796	323101284	3A	353561511	3B	225522075	3D
AX-94586703			360365511	3B	272515782	3D
AX-94383038	341284418	3A	343473137	3B	237831620	3D
AX-95155568			343481382	3B	237843414	3D
AX-94536028	260069628	3A	375251443	3B	241675687	3D
AX-94824311	321113870	3A	349049273	3B	244304184	3D
AX-94517678	365966470	3A	366097228	3B	274735161	3D
AX-94436244	367780692	3A	367227977	3B	281341035	3D
AX-94593481	367780797	3A	367227872	3B	281341140	3D
AX-94824776	386826890	3A	384080205	3B	292033774	3D
AX-94985457	418755461	3A	405128063	3B	310548486	3D
AX-94600301	449470756	3A	433022678	3B	336547556	3D
AX-94781891	454173293	3A	433287416	3B	336844768	3D
AX-95178690	456777350	3A	435358254	3B	338498433	3D
AX-95238487	458680810	3A	442422284	3B	344347340	3D
AX-94709681	485012301	3A	472623635	3B	363876360	3D

AX-94723713			486036357	3B	373609060	3D
AX-95155223	504807200	3A	495690864	3B	381641984	3D
AX-94814389	509998172	3A	505209351	3B	389083218	3D
AX-94541525	512309385	3A	509657459	3B	392062940	3D
AX-95137717	516554672	3A	520975004	3B	397145382	3D
AX-94933191	522060558	3A	525817290	3B	401683251	3D
AX-94821626	527938101	3A	529979870	3B	405380130	3D
AX-94457445	538313970	3A	539070897	3B	414651807	3D
AX-95108914	540237109	3A	542266637	3B	417219422	3D
AX-94416649	564349244	3A	553159034	3B	424658884	3D
AX-94848217	558891599	3A	555602028	3B	427454561	3D
AX-95231942	556669922	3A	559607901	3B	429103500	3D
AX-94395880	575001503	3A	570785853	3B	436682305	3D
AX-94575721	579347769	3A	574940045	3B	439670183	3D
AX-94540584	581263944	3A	576894378	3B	441144551	3D
AX-94870471	596799864	3A	596080247	3B	454764016	3D
AX-94829373	597164619	3A	596274627	3B	454900271	3D
AX-94824695	606425948	3A	614689914	3B	463089032	3D
AX-95191565	608340263	3A	616941159	3B	465028840	3D
AX-94774457	620607443	3A	634753924	3B	477020359	3D
AX-94798477	623061357	3A	638583225	3B	479636680	3D
AX-94417262	630792708	3A	648574326	3B	487132037	3D
AX-94741978	631584230	3A	649314382	3B	487822880	3D
AX-94854568	631584314	3A	649314466	3B	487822964	3D
AX-94736568	636535646	3A	657112243	3B	497877296	3D
AX-94552167	642959171	3A	665018851	3B	504984609	3D
AX-94445863			669035502	3B	508198686	3D
AX-94690389	649714360	3A	676249874	3B	515021317	3D
AX-95260889	662395465	3A	696395050	3B	527673517	3D
AX-95252784	662397331	3A	696396800	3B	527675218	3D
AX-94767744	663438065	3A	698564789	3B	529492855	3D
AX-95108342	680754082	3A	718273739	3B	543885409	3D
AX-94472908	683252479	3A	722001557	3B	546483470	3D
AX-94942170	688621490	3A	730808664	3B	551711915	3D
AX-95242795	692719179	3A	737248327	3B	556208291	3D
AX-94575205			743717564	3B	560679810	3D
AX-94643912	616966273	6A	717862948	6B	472722418	6D

Linkage group four rye bin map blast data

The markers are ordered according to the rye bin map and the IWGSC reference position is shown the chromosome with the highest blast result has been marked in green

Marker Name	IWGSC position	Chrm	IWGSC position	Chrm	IWGSC position	Chrm
AX-94921018	599325514	4A	7038401	4B	4354328	4D
AX-94852529	598399529	4A	8983315	4B	309953410	6D
AX-94767360	597909523	4A	53016546	Un	5593024	4D
AX-94771772	597693265	4A	10558756	4B	6019293	4D
AX-94456693	596920171	4A	11675578	4B	6495185	4D
AX-94960117	595984892	4A	13421042	4B	7648061	4D
AX-94644898	595984354	4A	13421563	4B	7648592	4D
AX-94868648	594213681	4A	17095038	4B	9216868	4D
AX-94400919	590114760	4A	23011181	4B	12462489	4D
AX-94678384	584273259	4A	28072389	4B	16234679	4D
AX-95232028	583525871	4A	28960052	4B	16923676	4D
AX-94868908	581482811	4A	31876393	4B	19408694	4D
AX-94467032	577756932	4A	37697866	4B	25441307	4D
AX-94562031	570992528	4A	45509597	4B	31810909	4D
AX-94519609	556775666	4A	62948564	4B	43277140	4D
AX-94565283	551219418	4A	68891784	4B	47254686	4D
AX-94656159	550957556	4A	69163050	4B	47438842	4D
AX-94975092	542271684	4A	81371512	4B	54799632	4D
AX-95150779	541172463	4A	82918919	4B	55645134	4D
AX-95114984	537437383	4A	87811974	4B	58391149	4D
AX-94424175	531241339	4A	95267456	4B		
AX-94859295	466928549	4A	171026023	4B	109909133	4D
AX-94492194	436829043	4A	54431132	Un	130466012	4D
AX-95068915	423587909	4A			136964218	4D
AX-95080921	210165711	4A	273244598	4B	275759591	4D
AX-94594232	187262338	4A	370425492	4B	298541374	4D
AX-94681393	182219259	4A	373981820	4B	300783411	4D
AX-94468909	181621592	4A	374729541	4B	301021779	4D
AX-95219068	178375519	4A	376423849	4B	301952000	4D
AX-94917775	255444321	1A	237486000	7B		
AX-94591682	236549649	7A	174542357	7B	222092772	7D
AX-94996861	217958561	7A	196631586	7B	205673747	7D
AX-94757080	206865620	7A	162087717	7B	197824394	7D
AX-94509195	195758348	7A	42911926	6B	188239381	7D
AX-95237806	187032176	7A	152769519	7B	183743168	7D
AX-94639884	187370196	7A	152457919	7B	183683693	7D
AX-95173422	184515546	7A	146026811	7B	180931426	7D

AX-94581390	182544195	7A	144634324	7B	178558924	7D
AX-94787077	150925461	7A	113178618	7B	151275644	7D
AX-94839360	139474947	7A	104745946	7B	139798851	7D
AX-95105797	129190080	7A	91636107	7B	130065639	7D
AX-95162927	118114809	7A	72312416	7B	112787781	7D
AX-94693251	118114795	7A	72312402	7B	112787767	7D
AX-95253527	117017220	7A	71591943	7B	112082983	7D
AX-94894854	101203263	7A	54753416	7B	98946966	7D
AX-95200460	94417660	7A	44814522	7B	92540472	7D
AX-94472180	93006240	7A	44133560	7B	91341104	7D
AX-94501130	91874567	7A	41778994	7B	90547497	7D
AX-95015721	85163534	7A	33081690	7B	83580003	7D
AX-94580723	83580240	7A	28503249	7B	80925148	7D
AX-94849528	83236175	7A	27822459	7B	80456720	7D
AX-95018673	82142933	7A	26748291	7B	79490036	7D
AX-94630793	65924479	7A	3689368	7B	63279766	7D
AX-95122633	665502789	4A			51475798	7D
AX-94700184	42076874	7A				
AX-94456809	42076676	7A			42649821	7D
AX-94901129	42076256	7A			42644491	7D
AX-95206728	35734203	7A			35558946	7D
AX-94726646	692547495	4A			33006655	7D
AX-94711978	692792422	4A			32644741	7D
AX-94803533	690421898	4A	82978458	Un	32452394	7D
AX-95223512	690421906	4A	82978464	Un	32452386	7D
AX-94676208	710175542	4A	351976016	Un	25274288	7D
AX-95165382	1442447	7A			11411037	7D
AX-94835523	4605429	7A	52142510	Un	7068612	7D
AX-95083227	13046778	6A	10913061	6D	20687221	6B
AX-94539061	2841380	6A			4410528	6D
AX-94593555	2974407	6A	7900263	6B	4265416	6D

Linkage group five rye bin map blast data

The markers are ordered according to the rye bin map and the IWGSC reference position is shown the chromosome with the highest blast result has been marked in green

Marker Name	IWGSC position	Chrm	IWGSC position	Chrm	IWGSC position	Chrm
AX-94623985	2315551	5A	1745534	5B	1451719	5D
AX-95251288					3031767	5D
AX-94418907					3031797	5D
AX-95165242	1604085	5A			7002930	5D
AX-94440407	6713693	5A	8917824	5B	8542779	5D
AX-94623475			16033800	5B	22441122	5D
AX-95151346	21409076	5A	24098168	5B	32693212	5D
AX-94510065	26460886	5A	27822304	5B	37355964	5D
AX-94672713			37058815	5B	42556270	5D
AX-94628315	71186074	3B	274351229	5B	46264566	3D
AX-95197582	39737680	5A	53024232	5B	50323434	5D
AX-94666338	40720512	5A	54702913	5B	51686697	5D
AX-95131464	41424287	5A	55371327	5B	52281443	5D
AX-94499088	42622279	5A	57437017	5B		
AX-94712036			58190586	5B		
AX-94697400	64959048	5A	81484123	5B	72500650	5D
AX-94509099					76203044	5D
AX-94596869			87975555	5B		
AX-94473146			87977300	5B	78119007	5D
AX-94737943	83278289	5A	94676549	5B	89381819	5D
AX-94729687	85819979	5A	97491697	5B	92141299	5D
AX-94642780	88646584	5A	101547138	5B	94343615	5D
AX-94458384	88989713	5A	101846485	5B	94500926	5D
AX-95177571	100619225	5A	108795952	5B	100034281	5D
AX-94666267	100619227	5A	108795954	5B	100034283	5D
AX-95123099	100619333	5A	108796060	5B	100034389	5D
AX-94477803	109279377	5A	115074793	5B	105103667	5D
AX-94957866	109279981	5A	115075397	5B	105104271	5D
AX-94772679	139927518	5A	138769828	5B	125802444	5D
AX-94955834	169299771	5A	147436473	5B	135658443	5D
AX-94480507	172519874	5A	185720961	5B	137053016	5D
AX-94439043	172461929	5A	185726798	5B	137058387	5D
AX-95183070	206725001	5A	194190529	5B	150829838	5D
AX-94590058	245953834	5A	176215529	5B	159657161	5D
AX-94486930	113608361	5A	157046362	5B	180488684	5D
AX-95098750	210477824	5A	153193903	5B	181883643	5D
AX-95219822	199216144	5A	161084971	5B	187960066	5D

AX-95099593	211639329	5A				
AX-94564318	242853634	5A	153503994	5B	188337715	5D
AX-94559227	176446127	5A	162494206	5B	170317449	5D
AX-94673656	227713242	5A	209645362	5B	198957715	5D
AX-94996150	265280516	5A	219817783	5B	206597337	5D
AX-95203559	268466966	5A			207782581	5D
AX-94995549	307352589	5A	254576274	5B	228779947	5D
AX-94944074	315494725	5A	266004155	5B	238712742	5D
AX-94686719	313906267	5A	263546471	5B	239940586	5D
AX-94601618	312891786	5A	262752947	5B	241020759	5D
AX-94485096			447531797	5B	251426166	5D
AX-94845600	335695758	5A	285831753	5B	253383913	5D
AX-94546491	341690970	5A	291088202	5B	256752412	5D
AX-94500822	356495812	5A	304624171	5B	268901259	5D
AX-94613034	369639263	5A	316868010	5B	278623711	5D
AX-94542162	398240110	5A	337905589	5B	297578985	5D
AX-94804111	392721715	5A	344610380	5B	301569578	5D
AX-94549978	399786079	5A	351461147	5B	306189083	5D
AX-94557815	399789528	5A	351475107	5B	306192040	5D
AX-94418705	410802679	5A	366008978	5B	316244943	5D
AX-94407767	415605528	5A	370193247	5B	319689738	5D
AX-94589335	416599769	5A	372094634	5B	320886486	5D
AX-94586263	437040880	5A	393845376	5B	335877561	5D
AX-95107006	437217706	5A	394016848	5B	336056516	5D
AX-94941864	451741525	5A	412462018	5B	351404193	5D
AX-94881794	476604617	5A			375193882	5D
AX-94952204	447531149	5B	476605265	5A	375194500	5D
AX-95102303	477435749	5A	449750883	5B	376662754	5D
AX-94863418	484080876	5A	460267765	5B	383778244	5D
AX-94585720	485203212	5A	461137594	5B	384344057	5D
AX-95146573	508872646	5A	486484737	5B	405440019	5D
AX-95240372	513845815	5A	489283310	5B	407967079	5D
AX-94506346	536870256	5A	511072742	5B	423371390	5D
AX-95117413	558671935	5A	538710554	5B	442489653	5D
AX-94666215	561533282	5A	541343030	5B	444549217	5D
AX-94618448	561555032	5A	541529367	5B	444712796	5D
AX-94764782	561555150	5A	541529485	5B	444712913	5D
AX-94724254	563170373	5A	542952763	5B	445319018	5D
AX-94648929	564079960	5A	544334664	5B	446405656	5D
AX-94882660	591318697	5A	578367874	5B	471047408	5D
AX-94727436	595037554	5A	582975167	5B	475575469	5D
AX-94508767	596448561	5A	584821846	5B	476940873	5D
AX-95182970	605100521	5A	594745230	5B	483645639	5D
AX-95153922	606018100	5A	595469188	5B	484308701	5D
AX-94876255	606787143	5A	597080466	5B	486031380	5D

AX-94450794	607681852	5A	599653848	5B	487289205	5D
AX-94934718	607682104	5A	599654099	5B	487289456	5D
AX-94407478	609865317	5A	601876822	5B	488855574	5D
AX-94868347	617356289	5A	610687609	5B	494467803	5D
AX-94932471	617356811	5A	610688173	5B	494468273	5D
AX-94560830	633863473	5A	636653508	5B	506096190	5D
AX-94497385	641731205	5A	644887966	5B	513975581	5D
AX-94700378	649781659	5A	657714408	5B	521401307	5D
AX-94570846	659879073	5A	612261416	4B	482691368	4D
AX-94895095	670627108	5A	627323209	4B	489179637	4D
AX-94447649	670821058	5A	627521309	4B	489188549	4D
AX-94805012	674123906	5A	634191806	4B	494819248	4D
AX-95008348	677845262	5A	638588786	4B		
AX-95160280	677845450	5A	638588974	4B		
AX-94602198	679149019	5A	640579764	4B	498613581	4D
AX-94551199	679664592	5A	641268004	4B	498897371	4D
AX-94587019	680742907	5A	642959741	4B		
AX-94621416	682711784	5A	645429379	4B	501822193	4D
AX-94638862	688321419	5A	649605846	4B	504173266	4D
AX-94500324	690186221	5A	652772578	4B		
AX-95097048	690186824	5A	652773181	4B		
AX-94861038	690190174	5A	652776508	4B		
AX-95141208	692061983	5A	654465596	4B	507120204	4D

Linkage group six rye bin map blast data

The markers are ordered according to the rye bin map and the IWGSC reference position is shown the chromosome with the highest blast result has been marked in green

Marker Name	IWGSC position	Chrm	IWGSC position	Chrm	IWGSC position	Chrm
AX-95185836	34980951	6A	64232118	6B	103793970	Un
AX-94620406	35633622	6A	65728399	6B	101799403	Un
AX-94468557	58150813	6A	94000056	6B	42564347	6D
AX-94663483	58150558	6A	93999801	6B	42564602	6D
AX-94784805	84922633	6A	142229560	6B	67377364	6D
AX-94661886	85591216	6A	143204534	6B	68103667	6D
AX-95218218	104052449	6A	163969106	6B	86546850	6D
AX-95161661	107244153	6A	171183483	6B	89157037	6D
AX-95143205	123536433	6A	188189414	6B	102467654	6D
AX-94880647	135245170	6A	199763563	6B	110882126	6D
AX-95215629	140605826	6A	202349704	6B	112940169	6D
AX-94454654	143965251	6A	204923415	6B	115545595	6D
AX-94701592	169514378	6A	228974797	6B	131608338	6D
AX-94461514	198365588	6A	261526531	6B	148767356	6D
AX-94874327	198364452	6A	261527672	6B	148768504	6D
AX-94584609	198364226	6A	261527821	6B	148768662	6D
AX-94786019	222695944	6A	299591767	6B	162971802	6D
AX-94876114	258141313	6A	309746964	6B	163836156	6D
AX-95193304	276310378	6A			206550098	6D
AX-94551285	319201903	6A	376289834	6B	239043975	6D
AX-94451291			530636543	7B	239048422	6D
AX-94548622	373995238	6A	408621034	6B	264669302	6D
AX-94487837	389246133	6A	433706441	6B	272648344	6D
AX-94407381	410893883	6A	470129793	6B	304288987	6D
AX-94419941	409091513	6A	471230084	6B	305358871	6D
AX-94946469	441499976	6A	473064761	6B	306137963	6D
AX-94765621	453908711	6A	508604085	6B	316101394	6D
AX-94747714	475262816	6A	484984194	6B	335984899	6D
AX-94925385	481259986	6A	479075580	6B	341198442	6D
AX-94528665	499399740	6A	538363008	6B	357005390	6D
AX-94554969	545127274	6A	597294400	6B	399035042	6D
AX-94868359	571021772	6A	641395705	6B	426101592	6D
AX-94970634	581734519	6A	657089650	6B	434442220	6D
AX-94800440	587556539	6A			438323843	6D
AX-94682046	587556796	6A				
AX-94927028	597289581	6A	685665646	6B	451657344	6D

AX-95211372			686172593	6B	451763554	6D
AX-94392250	597832398	6A	686416172	6B	451908926	6D
AX-94712310	597832405	6A	686416179	6B	451908933	6D
AX-95151713	600131154	6A	689523435	6B		
AX-95144058	609167003	6A	704879335	6B	452099203	6D
AX-94404191	609036026	6A	704452165	6B	462201639	6D
AX-94956710	710274780	3A	763196120	3B	575136571	3D
AX-94832124			778265221	3B	582029366	3D
AX-94827378			782371784	3B	587103832	3D
AX-95254452	729315150	3A			599274473	3D
AX-95216889	312075264	3A	810832225	3B	604679887	3D
AX-95096469	740300422	3A				
AX-94674014	740300282	3A				
AX-94630546	720839701	7A	723917712	7B	625163190	7D
AX-94588978	721032156	7A	725531301	7B	625481655	7D

Linkage group seven rye bin map blast data

The markers are ordered according to the rye bin map and the IWGSC reference position is shown the chromosome with the highest blast result has been marked in green

Marker Name	IWGSC position	Chrm	IWGSC position	Chrm	IWGSC position	Chrm
AX-94962192			588945	7B	559874652	5D
AX-95109523	603727097	4A	134809539	6B	558275643	5D
AX-95243342	629860533	4A	680359018	5B	538788128	5D
AX-94866959	625367506	4A	685301487	5B	543777209	5D
AX-95199123	625465489	4A			543688000	5D
AX-94879120	629476205	4A	680604225	5B	539025337	5D
AX-94694080	632858602	4A	677881090	5B	537393700	5D
AX-94689928	632858791	4A	677880899	5B	537393938	5D
AX-94590716	636971461	4A	674888215	5B	534619076	5D
AX-95165541	640003384	4A	671356286	5B	532247938	5D
AX-94490226	610484922	4A	704883334	5B	562158553	5D
AX-94638414	3149654	4A	586462965	4B	468210377	4D
AX-94876972	5464992	4A	581309280	4B	464988482	4D
AX-95191310	5465184	4A	581309088	4B	464988290	4D
AX-94751291	10636838	4A	572554940	4B	458385521	4D
AX-94972982	10938582	4A	572157673	4B	458091707	4D
AX-95101430	24757387	4A	552029697	4B	442904311	4D
AX-94835958	37825963	4A	526953663	4B	428569656	4D
AX-94531979	40435872	4A	520238872	4B	423907899	4D
AX-95149006	46123551	4A	515165866	4B	418617025	4D
AX-95108453	443327942	6A	500261230	4B	403403532	4D
AX-94683511	75426406	4A	483763119	4B	394144322	4D
AX-94460847	481424711	Un	479676850	4B	389575761	4D
AX-94997464	68565572	4A	475255719	4B	387085562	4D
AX-94739374	81764830	4A	469247817	4B	381899570	4D
AX-94819855	81822136	4A	469011516	4B	381878874	4D
AX-94516330	88080638	4A			377830201	4D
AX-94821047	114587185	4A	433646831	4B	350502307	4D
AX-94822221	140282744	4A	411532559	4B	333070609	4D
AX-94658455	263660684	7A	223155929	7B	246776759	7D
AX-94484014	275848795	7A	234567774	7B	255920134	7D
AX-94944053	292747222	7A	247236964	7B	266830331	7D
AX-94880343	354357386	7A	277626347	7B	345532773	7D
AX-95174833	425710663	7A	372802484	7B	378126008	7D
AX-94523738	427544937	7A	374538659	7B	379271510	7D
AX-94786798	436991523	7A	382247996	7B	387170607	7D

AX-95216565	446252008	7A	393291784	7B	393535150	7D
AX-94860725	446252037	7A	393291810	7B	393535179	7D
AX-94630310	446252053	7A	393291826	7B	393535195	7D
AX-94867730	446286390	7A	393369504	7B	393569456	7D
AX-95018320	472050139	7A	425448970	7B	410015472	7D
AX-94741120	473164100	7A	426500582	7B	412059175	7D
AX-95089647	473836775	7A	426855159	7B	412628930	7D
AX-95083230	474454246	7A	427369097	7B	413064186	7D
AX-94490071	513150778	7A	435781222	7B	419745660	7D
AX-94387172	505614531	7A	444994047	7B	426956880	7D
AX-95235263	490898698	7A	457375756	7B	439675317	7D
AX-95124586	495508008	7A	461220304	7B	444334289	7D
AX-94546581	545877842	7A	468825568	7B	451005022	7D
AX-95145478	543354720	7A	479031657	7B	454638819	7D
AX-94692189	540879313	7A	481043501	7B	459036038	7D
AX-94497485			485070129	7B	462094335	7D
AX-95226247	536436498	7A	487777456	7B	463848314	7D
AX-94828496	553310067	7A	512724599	7B	485052539	7D
AX-95120206	556430703	7A	518360179	7B	489675408	7D
AX-95235352	567504173	7A	530347356	7B	501045800	7D
AX-95216246	571629622	7A	532250319	7B	504132186	7D
AX-94730568			538362509	7B		
AX-94787776	581302669	7A	539193528	7B	510571344	7D
AX-94779493	581302669	7A	539193528	7B	510571344	7D
AX-94811369	592228186	7A	550640206	7B	517532442	7D
AX-94889553	596642613	7A	553598507	7B	520357632	7D
AX-94927921	608653272	7A	566605694	7B	529001540	7D
AX-95081313	612039478	7A	572007391	7B	531576385	7D
AX-94943283	641950721	7A	603235824	7B	556176752	7D
AX-94887824	645030251	7A	608875028	7B	560977809	7D
AX-94789814	645030322	7A	608875099	7B	560977882	7D
AX-95007493	654728943	7A	622236510	7B	567696535	7D
AX-94453227	659482605	7A	626203093	7B	570621909	7D
AX-94511236	663504060	7A	632755862	7B	574229030	7D
AX-94701057	666916548	7A	638450732	7B	576133266	7D
AX-94557270	667148047	7A	638879628	7B	576248707	7D
AX-94854111	14951505	2A	23188744	2B	12785879	2D
AX-95080496	13409095	2A	22317416	2B		
AX-94609984	687646621	5A	18824041	2B	12414732	2D
AX-94938434	10600706	2A	14047091	2B	10591701	2D
AX-94485004					8169908	2D
AX-94698374	2638935	2A	7428699	2B	3789254	2D

Preliminary SPAD and chlorophyll fluorescence data

	SPAD1	SPAD 2	SPAD 3	QY1	QY2	QY3	FLOWERING
CS Euploid	45.9	44.9	46.8	0.77	0.77	0.76	Pre antheisis
Paragon	47.7	44	46.2				Pre antheisis
BC2-306F	53.2	54.8	53.2	0.77	0.78	0	Pre antheisis
BC2-306B	58.4	58.1	48	0.77	0.78	0.8	Pre antheisis
BC2-306C	53.4	51.1	52.2	0.77	0.78	0.77	Pre antheisis
BC2-306D	51.5	51.5	50.8	0.76	0.74	0.77	Pre antheisis
BC2-306E	53	53	52.3	0.77	0.74	0.8	Pre antheisis
BC2-565C				0.77			Pre antheisis
BC2-728A				0.79			Pre antheisis
BC2-730A	54.9	49.3	50.1	0.77	0.8		Pre antheisis
BC2-731A	50.1	49.3	48.9	0.76	0.76	0.76	Pre antheisis
BC2-732A	50.4	47.6	48.2				Pre antheisis
BC2-733A	57.4	50.5	55.5	0.76	0.74	0.77	Pre antheisis
BC2-733B	42.6	44.1	43.1				Pre antheisis
BC2-734A	45.6	46.7	47.1	0.76	0.74	0.77	Pre antheisis
BC2-734C	56.9	55.5	55.3	0.71	0.78	0.75	Pre antheisis
BC2-736A	53.1	54.4	53	0.73	0.7	0.78	Pre antheisis
BC2-736B	44.7	46.6	49.3	0.79	0.79	0.78	Pre antheisis
BC2-736C	48.2	50.1	50	0.77	0.76	0.77	Pre antheisis
BC2-738A	43	46.3	42	0.79	0.76	0.78	Pre antheisis
BC2-738B	52.9	55.3	47.3	0.74	0.76	0.79	Pre antheisis
BC2-738D	60.7	55.7	62.3				Pre antheisis
BC2-739A	49.2	46.2	49.9				Pre antheisis
BC2-739B	50.3	48.7	42.7				Pre antheisis
BC2-739C	52.1	52.5	53				Pre antheisis
BC2-739D	46.5	44.8		0.75	0.77		Pre antheisis
BC2-739E				0.76	0.75	0.72	Pre antheisis
BC2F1-253A	43.4	44.6	42.6	0.72			Pre antheisis
BC2F1-253B							Pre antheisis
BC2F1-254A	48.6	50.7	50.8	0.77	0.75	0.76	Pre antheisis
BC2F1-254B	47.3	47.5		0.75	0.76		Pre antheisis
BC2F1-254C	56.5	52.7		0.75	0.79		Pre antheisis
BC2F1-254D	54.7	53	50.6	0.74	0.75	0.77	Pre antheisis
BC2F1-254E	54.4	51.5	49.9	0.76	0.78	0.76	Pre antheisis
BC2F1-254F	51.3	51.8	52	0.71	0.73		Pre antheisis
BC2F1-254G	53.6	50.7	52.6	0.75	0.76	0.7	Pre antheisis
BC2F1-254H							Pre antheisis
BC2F1-255A	37.4	47.4	47.1	0.74	0.67	0.72	Pre antheisis
BC2F1-255B	53.1	50.9	54.2	0.75	0.74	0.76	Pre antheisis
BC2F1-255C	53.8	50.6	54.8	0.78	0.75	0.63	Pre antheisis
BC2F1-255D	53.4			0.66			Pre antheisis
BC2F1-255E							Pre antheisis

BC2F1-257A							Pre antheisis
BC2F1-68A				0.77	0.74		Pre antheisis
BC2F1-68B				0.75			Pre antheisis
BC2F1-68C				0.78	0.77	0.76	Pre antheisis
BC3-351E	53.9	53.2	54	0.76	0.73	0.78	Pre antheisis
BC3-351F	50.3	49.8	50.2	0.75	0.77	0.74	Pre antheisis
BC3-351G	52.4	48.1	52.2	0.77	0.77	0.71	Pre antheisis
BC3-351H	53.9	48.9	54.9	0.77	0.75	0.71	Pre antheisis
BC3-635A	56.3	55.6		0.78	0.69		Pre antheisis
BC3-635B	54.6	51.6	53.1	0.78	0.75	0.77	Pre antheisis
BC3-636A	51.1	53	51				Pre antheisis
BC3-636B							Pre antheisis
BC3-636C	46	47.6	50.9				Pre antheisis
BC3-636D							Pre antheisis
BC3-636E	53	60.5	57.6	0.68	0.75	0.75	Pre antheisis
BC3-637A	49.6	44.9	50.1	0.79	0.78	0.77	Pre antheisis
BC3-637B	48.3			0.79			Pre antheisis
BC3-637C	38.9	48.4		0.74	0.75		Pre antheisis
BC3-637D							Pre antheisis
BC3-637E	51.1	50.1	55	0.77	0.79	0.79	Pre antheisis
BC3-638A	51.1	50.1	53	0.75	0.74	0.77	Pre antheisis
BC3-638B	45.4	47.4	46.2				Pre antheisis
BC3-638C	50	50	50.1	0.7	0.6	0.76	Pre antheisis
BC3-638D	49.2	46.4	48.2	0.73	0.6	0.75	Pre antheisis
BC3-638E	48.6	47	50.5	0.77	0.78	0.76	Pre antheisis
BC3-639A	56	55.8	50.5	0.62	0.79	0.77	Pre antheisis
BC3-639B	55.7	55.4	49.6	0.79	0.74	0.76	Pre antheisis
BC3-639C	48.7	48	48.3				Pre antheisis
BC3-639D	50.2	50.3					Pre antheisis
BC3-640A	56.1	53.9	54.9	0.78	0.78	0.74	Pre antheisis
BC3-640B	52.6	51.8	50.5	0.8	0.79	0.76	Pre antheisis
BC3-640C	48.4	48	47.9	79	0.78	0.78	Pre antheisis
BC3-640D				0.77			Pre antheisis
BC3-640E	50.1	49.3	50.2	0.75	0.7	0.75	Pre antheisis
BC3-640F	54.1	55.3	54.3	0.75	0.76	0.76	Pre antheisis
BC3-640G	54.8	52.3	52.5	0.77	0.75	0.72	Pre antheisis
BC3-643A	53.2	54.7	56.2	0.78	0.77	0.77	Pre antheisis
BC3-644A	54.8	55.7	55.7	0.77	0.75	0.72	Pre antheisis
BC3-644B	47.7	47.9	48.4	0.76	0.77	0.77	Pre antheisis
BC3-644C	50.8	53		0.73	0.74	0.78	Pre antheisis
BC3-645A	51	51.7	53.1	0.75	0.74	0.74	Pre antheisis
BC3-645B				0.77			Pre antheisis
BC3-645C	52.3	45.9	47.2	0.76	0.76	0.77	Pre antheisis
BC3-646B							Pre antheisis
BC3-648A							Pre antheisis

BC3-648C	44.1	44.1	45.3	0.77			Pre antheisis
BC3-648D	53.4	49.3	48.1				Pre antheisis
BC3-649A	50.7	53.3	52	0.8	0.75		Pre antheisis
BC3-649B	53.8	55.9	54.9	0.72	0.75	0.75	Pre antheisis
BC3-649C	48.7	50.6	51.6	0.78	0.79	0.8	Pre antheisis
BC3-653A							Pre antheisis
BC3-657A	49.5	51.7		0.79	0.8		Pre antheisis
BC3-658A	58.3	55.3	56.7	0.75	0.79	0.75	Pre antheisis
BC3-658B	49.4	50.5	50.5	0.76	0.77	0.77	Pre antheisis
BC3-659A	46.9	49.5	49.8	0.76	0.77	0.74	Pre antheisis
BC3-659B	43.5						Pre antheisis
BC3-659C	49.4	43.7	49.6	0.79	0.77	0.78	Pre antheisis
BC3-659D	49.8	51.2	49.8	0.71	0.75	0.77	Pre antheisis
BC3-661A	43.9			0.76	0.79		Pre antheisis
BC3-662A	56.2	48.9	45	0.75	0.75	0.75	Pre antheisis
BC3-662B	53.7	51.9	52	0.75	0.75	0.75	Pre antheisis
BC3F1-180B	55.9	53.1	58.3	0.59	0.78	0.77	Pre antheisis
BC3F1-181A	46.7	47		0.78	0.78	0.78	Pre antheisis
BC3F1-181B	46.6	44.9	45.2	0.77	0.77	0.77	Pre antheisis
BC3F1-181C							Pre antheisis
BC3F1-182A	51.7	52.2	51.1	0.78	0.76	0.79	Pre antheisis
BC3F1-182B	54.5	52.4	52.2	0.7	0.74	0.76	Pre antheisis
BC3F1-182C	44	45.5	45				Pre antheisis
BC3F1-182D	44.4	48.8		0.78	0.76		Pre antheisis
BC4-228A	41	42.2	42.8	0.76			Pre antheisis
BC4-228B	50.4	48.9	45.2	0.74	0.74	0.77	Pre antheisis
CSEuploid	53.8	49.3	50.1	0.67	0.74	0.76	Post anthesis
Paragon	53.5	50.8	49.9	0.77	0.75	0.72	Post anthesis
BC2-306F	56.6	54.4	52.2	0.72	0.73	0.77	Post anthesis
BC2-306B							Post anthesis
BC2-306C				0.77	0.73		Post anthesis
BC2-306D	51.7	51.7	52.9	0.74	0.76		Post anthesis
BC2-306E	62.6	63.3	62.2	0.72	0.74	0.74	Post anthesis
BC2-565C	48	50.5	49.1	0.77	0.75	0.74	Post anthesis
BC2-728A	58.2	57.7	55.2				Post anthesis
BC2-730A	59.5	45.9	50.1	0.7	0.79	0.73	Post anthesis
BC2-731A	53.1	53.6	54.1	0.75	0.76	0.78	Post anthesis
BC2-732A	49.7	50	50.3	0.78	0.76	0.74	Post anthesis
BC2-733A				0.75	0.7		Post anthesis
BC2-733B	38.9	40.1	37.1	0.7	0.72		Post anthesis
BC2-734A	48.4	56.2	56.5	0.67	0.74		Post anthesis
BC2-734C	57.3	56.2	56.5	0.8	0.74		Post anthesis
BC2-736A	55.7	54.7	51.7	0.76			Post anthesis
BC2-736B	46.6	48.7	47.2	0.78	0.78	0.79	Post anthesis

BC2-736C	50.4	47.8	51.2	0.78	0.76	0.78	Post anthesis
BC2-738A	46.4	46.2	53.2	0.76	0.78	0.78	Post anthesis
BC2-738B	58.1	57.9	54.3	0.76	0.78	0.74	Post anthesis
BC2-738D	60	59.9	59.8	0.78	0.76	0.74	Post anthesis
BC2-739A	49.7	50.3	50.9	0.72	0.73	0.73	Post anthesis
BC2-739B	50.2	60.8	55.1	0.73	0.73	0.73	Post anthesis
BC2-739C	52.3	52.2	52.4	0.79	0.74	0.75	Post anthesis
BC2-739D							Post anthesis
BC2-739E							Post anthesis
BC2F1-253A				0.78	0.76	0.78	Post anthesis
BC2F1-253B							Post anthesis
BC2F1-254A	58.8	53.2	54	0.67	0.76	0.75	Post anthesis
BC2F1-254B	44.1	46.2	42.1	0.77	0.76	0.76	Post anthesis
BC2F1-254C	58.8	56.9	58.2	0.67	0.71		Post anthesis
BC2F1-254D	53.1	54.7	47	0.74	0.71	0.75	Post anthesis
BC2F1-254E	536	55.9	54.8	0.77	0.75	0.74	Post anthesis
BC2F1-254F	55.1	52.4	52.2	0.75	0.72	0.78	Post anthesis
BC2F1-254G	51.8	54.6	53.5	0.74	0.76	0.74	Post anthesis
BC2F1-254H							Post anthesis
BC2F1-255A	50.8	47.3	38	0.73			Post anthesis
BC2F1-255B	49.7	56.5	54.4	0.76	0.75	0.77	Post anthesis
BC2F1-255C	53.1	53	53.3	0.78	0.79	0.73	Post anthesis
BC2F1-255D	54.2						Post anthesis
BC2F1-255E							Post anthesis
BC2F1-257A							Post anthesis
BC2F1-68A	63	52.1	53	0.77	0.73	0.7	Post anthesis
BC2F1-68B	55.3	52.9	57	0.79	0.79	0.79	Post anthesis
BC2F1-68C	54.2	49.4	50	0.68	0.74	0.77	Post anthesis
BC3-351E	54.7	53.8	53	0.73	0.76	0.78	Post anthesis
BC3-351F	48	51.7	52	0.8	0.79	0.8	Post anthesis
BC3-351G	48	54.1	54.3	0.76	0.77	0.72	Post anthesis
BC3-351H	53.1	52	56.8	77			Post anthesis
BC3-635A	55.3	55.3	55.8	0.64			Post anthesis
BC3-635B	58	50.4	54.7	0.72	0.74	0.73	Post anthesis
BC3-636A	59.3	53	47.3				Post anthesis
BC3-636B							Post anthesis
BC3-636C	53.8	54.5	53.2	0.7	0.77	0.71	Post anthesis
BC3-636D	53.8	54.5	53.2	0.77	0.76	0.79	Post anthesis
BC3-636E							Post anthesis
BC3-637A	46.9	51.7	52	0.7	0.74	0.79	Post anthesis
BC3-637B	45	46.1	48.2	77	0.74	0.76	Post anthesis
BC3-637C	47.7	48.4	48	0.73	0.75		Post anthesis
BC3-637D							Post anthesis
BC3-637E	57.7	46.8	47.3	0.75	0.78	0.79	Post anthesis
BC3-638A				0.7	0.76	0.79	Post anthesis

BC3-638B	55	52.5	50.1				Post anthesis
BC3-638C				0.7	0.75	0.7	Post anthesis
BC3-638D	51.2						Post anthesis
BC3-638E							Post anthesis
BC3-639A	57.8	52.6	56.9	0.79	0.69	0.75	Post anthesis
BC3-639B	56.6	52.2	55.2	0.72	0.77	0.77	Post anthesis
BC3-639C							Post anthesis
BC3-639D							Post anthesis
BC3-640A				0.78	0.77	0.78	Post anthesis
BC3-640B	58.8	56.4	55.4	0.75	0.77	0.79	Post anthesis
BC3-640C	52.39	52.1	50.4	0.75	0.77	0.74	Post anthesis
BC3-640D	50.4			0.75	0.72	0.76	Post anthesis
BC3-640E	54.1	60.1	63.8	0.65	0.69	0.74	Post anthesis
BC3-640F	67.1	66.7	65.7	0.75	0.76	0.75	Post anthesis
BC3-640G	62.3	61.8	60.3	0.73	0.75	0.76	Post anthesis
BC3-643A	60.3	64.3	60	0.78	0.79	0.78	Post anthesis
BC3-644A	57.6			0.74	0.75	0.72	Post anthesis
BC3-644B	51.2	51.2	52.4	0.76	0.74	0.76	Post anthesis
BC3-644C	52.3	51.8	52.8	0.72	0.71	0.71	Post anthesis
BC3-645A	51.2	51.8	51.6	0.76	0.76		Post anthesis
BC3-645B	51.4	59.3	50.8	0.7			Post anthesis
BC3-645C	47.7	48.3	47	0.74	0.77	0.75	Post anthesis
BC3-646B							Post anthesis
BC3-648A	55.9	54.2	55.9	0.75	0.74	0.75	Post anthesis
BC3-648C	46.5	46.1	46.3	0.75	0.78	0.75	Post anthesis
BC3-648D	49.4	54.3	54.2	0.71	0.72	0.74	Post anthesis
BC3-649A	50.5	51.5	50.5	0.76	0.78	0.77	Post anthesis
BC3-649B	55.6	58.6		0.75	0.77	0.78	Post anthesis
BC3-649C	53.4	52.9	50.5	0.76	0.76	0.77	Post anthesis
BC3-653A				0.78	0.68		Post anthesis
BC3-657A	55.8	66.9		0.75	0.73	0.71	Post anthesis
BC3-658A	60.5	59.1	58.1	0.74	0.71	0.7	Post anthesis
BC3-658B	60.2	54.4	54.3	0.74	0.71	0.76	Post anthesis
BC3-659A	55.4	50.2	48.5	0.74	0.71	0.76	Post anthesis
BC3-659B							Post anthesis
BC3-659C							Post anthesis
BC3-659D	48.6	49.2	48.1	0.77	0.76	0.74	Post anthesis
BC3-661A	40.1	44.8	42.1	72	0.75	0.74	Post anthesis
BC3-662A	57.4	58.1	57.8	0.74	0.78	0.77	Post anthesis
BC3-662B	52.4	53.5	57.8	0.74	0.78	0.77	Post anthesis
BC3F1-180B	52.4	53.6	51.9	0.77	0.76	0.74	Post anthesis
BC3F1-181A	50.5	52	54.3				Post anthesis
BC3F1-181B	47.5	57.8	53.7				Post anthesis
BC3F1-181C	51	51.7	52	0.7	0.71	0.73	Post anthesis
BC3F1-182A	50.7	58.5	57.1	0.71	0.72	0.74	Post anthesis

BC3F1-182B	52.6	53.1	51.8	0.72	0.72	0.74	Post anthesis
BC3F1-182C							Post anthesis
BC3F1-182D	49.9	52.8	56	0.65	0.74	0.76	Post anthesis
BC4-228A	47	46	47	0.76	0.75	0.76	Post anthesis
BC4-228B	55.9	56.1	58.3	0.65	0.74	0.76	Post anthesis