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ESTABLISHMENT OF A TRACELESS GENOME EDITING SYSTEM IN WHEAT USING WHEAT X BARLEY HYBRIDIZATION

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Contents

1. ABBREVIATIONS

MPCR – Multiplex PCR

PCR – Polymerase Chain Reaction

ISH – *In Situ* Hybridization

GISH – Genome *In Situ* Hybridization

FISH – Fluorescence *In Situ* Hybridization

DNA – Deoxyribonucleic Acid

RNA – Ribonucleic Acid

GC – Guanine-Cytosine

BLAST – Basic Local Alignment Search Tool

F1 – First Filial Generation

F2 – Second Filial Generation

GM – Genetically Modified

WT – Wild Type

QTL – Quantitative Trait Locus

DH – Doubled Haploid

Bp – Base Pair

IWGSC – International Wheat Genome Sequencing Consortium

EFSA – European Food Safety Authority

DMSO – Dimethyl Sulfoxide

NaOCl – Sodium Hypochlorite

ATP – Adenosine Triphosphate

FAO – Food and Agriculture Organization

PPFD – Photosynthetic Photon Flux Density

MS – Murashige and Skoog Medium

BC – Back Cross

SDIS- Seed Development Induction Solution

EDTA – Ethylenediaminetetraacetic Acid

TBE – Tris/Borate/EDTA

CTAB – Cetrimonium Bromide

HCl – Hydrogen Chloride

PFA – Paraformaldehyde

SSC – Saline Sodium Citate

DAPI – 4′,6-diamidino-2-phenylindole

2. INTRODUCTION

The ever-increasing human population has led to a rise in cereal and food grain consumption of up to 70% worldwide, which complicates efforts to achieve food security by 2050. Wheat is currently farmed on more than 218 million acres worldwide, and its global trade is larger than the sum of all other crops (Kumar et al., 2022). Wheat plays a vital part in human nutrition and provides 20% of the daily protein and calorie intake from food. Wheat is counted among the 'big three' cereal crops, including rice and maize (Shewry, 2009).

Like other crops wheat productivity is threatened by numerous diseases and global climate change (Miedaner & Juroszek, 2021). Although, human selection has consistently enhanced agronomic traits in the allohexaploid wheat genome (2n=6x=42, AABBDD). However, due to the restriction of homoeologous pairing and recombination between its sub-genomes, the full potential of allelic diversity remains untapped, limiting its use for wheat improvement. Interspecific hybridization offers a promising strategy to boost genetic diversity and introduce traits such as resistance to biotic and abiotic stresses (Tonosaki et al., 2016). Hybridization between wheat and barley (*Hordeum vulgare* L., 2n=2x=14, HH genome) holds the potential to introduce valuable agronomic traits into wheat through chromosome addition, translocation lines, or even full hybrids (Molnár-Láng et al., 2014a).

In interspecific hybridization breeding, reproductive barriers that preserve species' genetic identity or restrict gene flow between species present significant challenges to successful hybrid development. One commonly observed phenomenon in wide crosses is the complete or partial elimination of one parent's genome during the early mitotic divisions of embryogenesis, particularly in distantly related interspecific and intergeneric hybrids (Ishii et al., 2016). In hybrids within the *Triticeae* tribe, this uniparental genome elimination typically affects the paternal genome (Bennett et al., 1976). Both complete and incomplete genome elimination hold substantial relevance for breeding strategies: while full elimination can generate maternal haploids that can be doubled to produce homozygous lines, partial or full hybrids serve as pre-breeding material,

enabling the non-GMO introgression of desirable traits such as disease resistance or improved agronomic performance (Polgári et al., 2019).

In this context, genome editing technologies such as CRISPR/Cas9 offer promising new avenues to complement or overcome the limitations of conventional technologies. The CRISPR/Cas9 system has emerged over the past decade as a precise and versatile tool for targeted genome modification, capable of editing multiple genes through small RNA guidance (Doudna and Charpentier, 2014). Initially identified in *Escherichia coli* as part of an adaptive immune mechanism (Ishino et al., 1987), its biological significance was later demonstrated in *Streptococcus thermophilus*, which incorporates viral DNA into CRISPR loci to confer phage resistance (Barrangou et al., 2007). The system functions by creating a double-stranded break in DNA, directed by a CRISPR RNA (crRNA) and a Cas protein (Schiml and Puchta, 2016). The resulting break is repaired via either the error-prone non-homologous end joining (NHEJ) pathway, which often disrupts gene function, or the more precise homology-directed repair (HDR) mechanism using a donor template (Belhaj et al., 2015).

However, many crop varieties are recalcitrant to the delivery of CRISPR/Cas9 using conventional methods like *Agrobacterium*-mediated transformation (Char et al., 2017; Lowe et al., 2016). Recent advancements in delivery systems include the overexpression of morphogenetic regulators such as BABY BOOM and WUSCHEL to improve tissue culture amenability, as well as the direct delivery of Cas9-sgRNA ribonucleoprotein complex into protoplasts to avoid consumer concerns and legal hurdles with transgenes. Despite these developments, these methods remain time-consuming, expensive, and often dependent on the specific genotype (Woo et al., 2015a). To address these challenges, technologies like Hi-Edit have been introduced, combining haploid induction with site-directed mutagenesis. This approach has been applied to A*rabidopsis* and in crops like maize, and wheat (Kelliher et al., 2019). However, reports in the literature indicate low editing efficiency, and no studies have documented wheat genome duplication following mutagenesis.

Hence, the hybridization of wheat barely holds the promise of transferring agronomically useful genes and gives an opportunity for the development of a DNA free site mutagenesis

technology in wheat by combining CRISPR/Cas9 technology and crossing of wheat and barley. Following the hybridization of wheat and barley, 20-90% of F1 plants may either be maternal haploid wheat or contain various combinations of wheat and barley chromosomes. Because of random chromosome composition it is necessary to determine the chromosome composition of F1 plants and their progenies (Barclay, 1975; Koba et al., 1991; Koba & Shimada, 1992; Polgári et al., 2014; Ali et al., 2024).

Thus, the above considerations and literature data prompted us to study the feasibility of the development of a DNA free technology for precision mutagenesis in wheat. Additionally, to develop a Multiplex PCR-based technology for rapid and cost-effective determination of the chromosome composition of wheat and barley. To check the applicability of this system on different wheat and barley species. And as a practical application test them on wheat and barley hybrids. To assess the taxonomic range and broader applicability of this system, test them on wild relatives and progenitor species of wheat (*Triticum*) and barley (*Hordeum*). To achieve this, a bioinformatics workflow needs to be developed which can be used to design chromosome-specific primers for wheat, barley and other agriculturally important species where interspecific hybridization is applied for genetic improvement.

3. LITERATURE REVIEW

3.1 The evolution and genetic background of wheat

Wheat species, regardless of their ploidy levels, share a basic genome structure consisting of seven chromosomes (x = 7). These ploidy levels can be diploid (2n = 2x = 14), tetraploid (2n = 4x = 28), or hexaploid (2n = 6x = 42), depending on the species within the genera *Triticum* and *Aegilops* (Kimber & Sears, 1987). The evolution of modern hexaploid bread wheat (*Triticum aestivum* L.) resulted from contributions from three diploid wild ancestors: *Triticum urartu* Tumanian ex Gandylian, an unidentified relative of *Aegilops speltoides* Tausch, and *Aegilops tauschii* Coss. These species contributed the three genomes (A, B, and D) found in modern bread wheat. The genetic relationship between these progenitor species and other grasses in the *Triticeae* tribe stems from both shared ancestry and hybridization events that occurred during wheat's evolution (Haas et al., 2019).

The A genome of hexaploid wheat originated from *T. urartu* (A^uA^u), while the B genome was derived from a member of the sitopsis group of *Aegilops* species, though the exact contributor is still debated. In the initial hybridization event, the A genome was provided by the pollen donor and the B genome by the egg donor. This hybridization gave rise to tetraploid wheat with a genome formula of BBAA (though some authors prefer AABB). The result of this hybridization was wild emmer wheat (*T. turgidum ssp. dicoccoides*), which later evolved into cultivated tetraploid emmer wheat (*T. dicoccum*), eventually giving rise to durum wheat (*T. turgidum ssp. durum*) (Figure 1) (Dvorak et al., 1993).

The emergence of hexaploid bread wheat occurred through a second hybridization event between free-threshing tetraploid emmer wheat (*Tg-A1/Tg-A1; Tg-B1/Tg-B1*) and *Aegilops tauschii* (*Tg-D1/Tg)*, which contributed the D genome (Figure 1). Early hexaploid wheat was likely hulled due to the presence of tenacious glumes (*Tg-D1*) from *Ae. tauschii*. Over time, domesticated wheat developed free-threshing characteristics, an essential trait for cultivation and grain processing. This shift was controlled by two main genetic factors: the *Tenacious glumes* (*Tg*) genes

and the *Q* locus on chromosome 5A. In hexaploid wheat, two homologous *Tg* genes (*Tg-B1* and *Tg-D1*) control the hull formation, while a third gene, *Tg-A1*, has been proposed based on molecular evidence but requires further study to confirm its role in domestication (Haas et al., 2019).

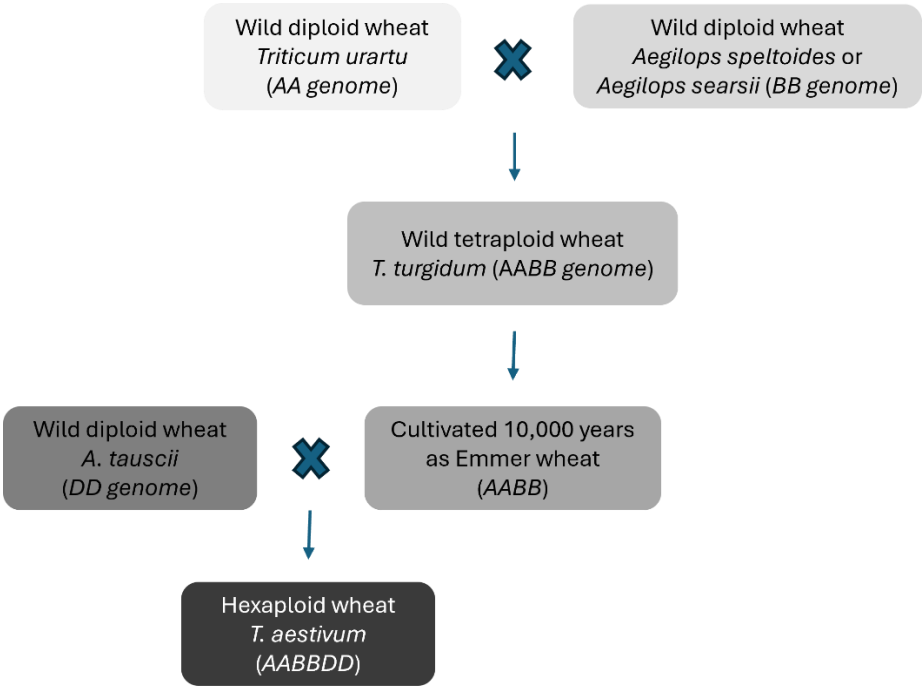

Figure 1: Illustrates the evolutionary and domestication history of wheat. Several diploid species contributed to the genetic makeup of modern wheat. A polyploidization event between *Triticum urartu* (AA genome) and a species from the sitopsis group, likely *Aegilops speltoides* (BB genome), produced *Triticum dicoccoides* (wild emmer; AABB). This wild emmer then gave rise to domesticated species such as *Triticum dicoccum* (domesticated emmer; AABB) and *Triticum durum* (durum wheat; AABB). The hexaploid wheat species, *Triticum aestivum* ssp. *aestivum* (bread wheat; AABBDD), originated from a cross between domesticated emmer (AABB) and *Aegilops tauschii* (goat grass; DD genome). The origins of hexaploid *Triticum aestivum* ssp. *spelta* (spelt; AABBDD) are debated, but it is likely not a direct ancestor of bread wheat and may instead result from hybridization between bread wheat and an emmer species (evolutionary outline adapted from Haas et al., 2019)

The *Q* locus on chromosome 5A is a key factor in wheat domestication, controlling the free-threshing trait and other agronomic features like spike shape, culm height, and rachis strength. The domesticated *Q* allele, a gain-of-function mutation, confers a free-threshing, square spike phenotype, while the wild-type *q* allele leads to elongated, speltoid spikes; however, the current *Q*

allele may have a weaker impact than the ancestral form (Salamini et al., 2002). In einkorn wheat, one of the earliest domesticated species, free threshing did not naturally occur. Although some soft-glumed (*sog*) einkorn accessions have been identified, the *sog* gene, mapped to chromosome 2A, is not orthologous to the *Tg* genes found in other wheats. It has been suggested that the *sog* mutation reduced ear length and yield potential, limiting the agricultural success of soft-glumed einkorn. In contrast, polyploid wheats, such as tetraploid and hexaploid species, avoided this drawback through genetic buffering, supporting greater robustness and productivity (Salamini et al., 2002).

Bread wheat (*Triticum aestivum* L.), an allohexaploid (2n=6x=42), undergoes meiosis, forming 21 bivalents (21 pairs of homologous chromosomes). These chromosomes are organized into three genomes, A, B, and D, with each genome containing seven chromosome pairs. The chromosomes of hexaploid wheat are be numbered from 1A, 1B, and 1D until 7A, 7B, and 7D, based on their genomic group. This classification was developed based on the studies of Hegde and Waines (2004) and earlier work by Longwell and Sears (1954). In hexaploid wheat, each chromosome has a homologous counterpart in the other two genomes, but a special gene called *Ph1*, located on chromosome 5B, prevents pairing between homeologous chromosomes (from different genomes) during meiosis (Sears, 1976; Feldman, 1993). This gene allows only homologous chromosomes (from the same genome) to pair, ensuring the stability of the wheat genome during reproduction. The *Ph1* gene has been critical in maintaining the genetic integrity of wheat, as it blocks homeologous pairing in hybrids with related species (Rey et al., 2018).

Interestingly, in hybrids between bread wheat and diploid *Aegilops* species, the expression of *Ph1* is suppressed, allowing homeologous chromosomes to pair. This phenomenon is important in wheat breeding, particularly when crossing wheat with its wild relatives to introduce new traits. Understanding the role of *Ph1* is also essential for studying evolutionary relationships among wheat species and their relatives (Riley et al., 1958).

Overall, the evolution of modern wheat has been shaped by a series of complex hybridization events involving its wild ancestors and the genetic contributions of various gene loci that control key domestication traits. The intricate interplay between these genetic factors has

allowed wheat to become one of the world's most important cereal crops, capable of adapting to a wide range of environmental conditions and agricultural practices (Matsuoka, 2011).

3.2 The evolution and genetic background of barley

Barley ranks fourth among grain cereals (*Poaceae* species) after maize (*Zea mays*), wheat (*Triticum aestivum*), and rice (*Oryza sativa*) in terms of global production. Barley is self-pollinating with a diploid genome consisting of seven chromosomes (2n = 2x = 14, HH) (Gaut, 2002). The estimated barley genome size is 5.1 Gbp9 with >80% of repetitive elements (Middleton et al., 2013). Cultivated barley (*Hordeum vulgare* L. ssp. *vulgare*) is remarkably morphologically similar to its wild progenitor, *Hordeum vulgare* ssp. *spontaneum*) (Figure 2). Both wild and domesticated barley were found in archaeological sites in the Fertile Crescent dating back about 10,000 years, which is believed to be the origin of barley domestication (Haas et al., 2019).

Figure 2: Wild barley (Left), Middle spike is two-rowed domesticated barley, and six-rowed domesticated barley on the right (Reproduced with permission from Dr. Martin Mascher, "Domestication and crop evolution of wheat and barley: Genes, genomics, and future directions," JIPB, 2019. Permission granted on 20 May 2025; see Appendices for a copy of the correspondence) (Haas et al., 2019).

There are three cytotypes in the genus *Hordeum*: diploid (2n = 2x = 14), tetraploid (2n = 4x = 28), and hexaploid (2n = 6x = 42), and the chromosomal number of all 32 species in this genus is n = 7. The *H* genome is shared by the vast majority of *Hordeum* species. Only *H. vulgare*

subsp. *vulgare* has been domesticated out of the 32 species that make up the genus *Hordeum* (Bothmer et al., 1985), becoming one of the most significant crops. Q. Chen (2005) proposed a reference sequence based on a map for the barley genome, which includes the first assembly of the pericentromeric portions of a *Triticeae* genome that is completely organized. The resource emphasizes a clear contrast between chromosomal distal and proximal regions, which is reflected in the arrangement of intranuclear chromatin. The seven chromosomes of barley were classified from 1 to 7 based on homoeologous relationships with the chromosomes of other species in the tribe Triticeae during the 7th International Barley Genetics Symposium held in Saskatoon, Saskatchewan, Canada, in 1996. This designation places a letter *H* after the chromosome number, for example, 2H. The letter *H* is used to represent the genomes of *H. vulgare* and *H. bulbosum* (https://digitalcommons.usu.edu/herbarium_pubs/20/).

In terms of evolution, the non-brittle rachis trait is considered one of the most significant adaptations in barley domestication, as it enhances harvesting efficiency by reducing seed loss. In contrast to brittle rachis spikes, which detach easily, the spikes of non-brittle plants stay on the plant even after maturation, making them more desirable for early agricultural harvest practices (Asfaw & Bothmer, 1990). This domestication trait marks a transition from wild seed dispersal mechanisms, which are vital for survival in nature, to those better suited for controlled farming. The earliest archaeological evidence of non-brittle barley originates from Tell Abu Hureyra, around 9500 BP (Hillman et al., 1989). Barley dispersal mechanisms include brittle and weak rachis forms (Kandemir, 2004). For wild barley species (*Hordeum*), spikes tend to disarticulate above each rachis node, producing wedge-shaped spikelets that exhibit smooth scars conducive to dispersal (Bothmer et al., 1995). In cultivated barley, however, threshing causes rougher dehiscence scars on grains, which detach from the rachis segments. Anatomically, brittle rachis nodes constrict sharply, while non-brittle rachis nodes do not exhibit such constrictions (Ubisch, 1915). The transition to non-brittle rachis was made possible by mutations in two key genes, *btr1* and *btr2*, both recessive and located on the short arm of chromosome 3HS (Takahashi & Hayashi, 1964; Komatsuda & Mano, 2002). Additional brittle-rachis genes are present in related species like *Triticum* and *Aegilops* across similar chromosome groups (W. Li & Gill, 2006; Watanabe & Ikebata, 2000).

One of the other significant adaptations was the shift from two-rowed to six-rowed barley, which substantially increased seed production per spike, a critical agronomic advancement. Six-rowed barley likely originated as a mutation of the ancestral two-rowed form, supported by genetic and archaeological evidence such as barley remains from Tell Abu Hureyra dating back to 8800 BP (Harlan et al., 1973). This change is unique to the genus *Hordeum*, whose ancestral two-rowed spike arrangement adapted over time to fertile six-rowed spikelets in domesticated forms (Bothmer & Jacobsen, 1985; Bothmer et al., 1995). However, ancestral two-rowed types are still bred, especially for malting or human consumption. For example, *Hordeum vulgare* cultivar 'Pinnacle' is a popular two-rowed barley used in brewing due to its uniform kernel size. This variety has high yield, low protein, and strong straw strength. Genetically, multiple loci contribute to the six-rowed trait, with the primary gene, *six-rowed spike* (*vrs1*), located on chromosome 2H long arm, controlling lateral spikelet fertility in cultivated barley. The *vrs1* gene's recessive allele enables six-rowed development, while the dominant allele maintains a two-rowed structure, found in wild barley (Lundqvist, 1997). Variations of this gene include alleles *vrs1.a* and *vrs1.c*, differing in lateral spikelet awn lengths. Additional loci, such as *vrs2*, *vrs3*, and *vrs4* on chromosomes 5HL, 1HL, and 3HL, respectively, further influence the degree of lateral spikelet fertility, though these are primarily found in mutant lines rather than cultivars (Lundqvist et al., 1997). The intermediate spike type, observed in two-rowed cultivars, is regulated by *six-rowed spike 5* (*vrs5*), a gene located on chromosome 4H short arm. This gene modulates fertility in lateral spikelets through alleles that either inhibit or allow partial seed development. Both *vrs1* and *vrs5* loci were crucial in the evolutionary pathway toward six-rowed barley, with the *vrs1* recessive mutation playing a pivotal role in barley domestication (Lundqvist & Lundqvist, 1987).

The presence of a hulled or naked caryopsis in barley is another important agronomic trait due to its impact on dietary use. Hulled barley retains a husk that adheres tightly to the grain, while naked barley has husks that separate easily during threshing. Archaeological findings of naked barley grains in Ali Kosh from around 8000 BP suggest that the mutation responsible for this trait appeared early in barley domestication (Helbaek, 1969). The transition to non-brittle rachis likely occurred before the naked caryopsis trait (Harlan, 1995). Genetically, a single recessive gene, *nud*, located on chromosome 7HL, controls the naked caryopsis, indicating that husk separation results

from a gene mutation (Fedak et al., 1972; Scholz, 1955). This trait is especially prevalent in East Asian regions such as Tibet, Nepal, and northern India, though historical evidence suggests it was also cultivated in ancient Anatolia and northern Europe (Helbaek, 1969). Genetic analysis of the *nud* gene supports a monophyletic origin, with studies showing a shared allele among 100 naked barley cultivars, distinguishing them from hulled and wild barley groups (Taketa et al., 2004).

Seed dormancy in barley enables survival through adverse conditions but poses issues in commercial contexts where rapid germination is required, especially in malting. This trait is influenced by genes and environmental interactions, with major quantitative trait loci (QTL) *SD1* and *SD2* on chromosome 5H playing key roles. *SD1*, near the centromere of 5H, is dominant and exerts the most significant influence on dormancy, while *SD2*, located on the long arm of 5H, controls moderate dormancy and is valuable for breeding programs (F. Han et al., 1996; C. Li et al., 2004).

Reduced vernalization requirements, essential for barley's adaptation to spring sowing, allow it to bypass the need for extended cold exposure before flowering. This change relies on three main genes: *Vrn-H1*, *Vrn-H2*, and *Vrn-H3* in barley. Wild barley is typically winter-hardy, but these genes enable a spring growth habit, particularly through a mutation in *Vrn-H2*, which arose independently multiple times according to molecular analysis. Comparative analysis shows that barley and wheat vernalization genes function similarly, reflecting shared evolutionary responses to seasonal change (Laurie, 1995; Yan et al., 2003, 2006).

Photoperiod insensitivity has been another crucial adaptation, allowing barley to flower independently of day length. Wild barley, which flowers earlier under long days, evolved in cultivated forms to tolerate an extended vegetative growth period. This adaptation is primarily controlled by the *Ppd-H1* gene on chromosome 2HS; the recessive *ppd-H1* allele is associated with insensitivity, favoring earlier flowering under long days. Another gene, *Ppd-H2*, on chromosome 1H, has a distinct role under short-day conditions, further expanding barley's adaptability across regions (Laurie, 1997; Turner et al., 2005).

3.3 Importance of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) cultivation

Wheat (*Triticum spp*. L) and barley (*Hordeum vulgare* L.) are two important cereal crops belonging to the *Poaceae* family. Wheat is a staple food for around 40% of the global population, while barley is primarily used for animal feed and brewing, although it remains an essential food source in regions where other cereals struggle to grow. Wheat's cultivation dates back thousands of years, making it one of the earliest domesticated crops. For about 8,000 years, wheat has played a central role in the diets of civilizations across Europe, West Asia, and North Africa, thanks to its adaptability, ease of storage, and the simplicity of converting it into flour for various food products (Percival, 1921). Today, wheat is the most widely grown crop in the world, covering more than 218 million hectares, with global trade surpassing that of all other crops combined. It contributes 20% of daily calories and protein intake for humans and supports the livelihoods of approximately 80 million farmers worldwide, making it the second most important food crop after rice in the developing world (Hanson, 1983). The vast majority (90-95%) of wheat production comes from common or bread wheat (*Triticum aestivum*), which is classified into hard and soft wheat based on grain hardness. This wheat is primarily milled into flour for a wide variety of leavened breads, flatbreads, and other baked goods. Durum wheat (*T. turgidum*), making up around 35–40 million tones of global production, is adapted to hot, dry climates, particularly around the Mediterranean, and is mainly used for pasta production. It is also referred to as "pasta wheat" or "durum wheat." In some regions, it is milled into flour for bread and used in couscous production (Bhattacharya & Corke, 1996).

Other, less common wheat species, such as einkorn (*T. monococcum*), emmer (*T. turgidum* var. *dicoccum*), and spelt (*T. aestivum* subsp. spelta), are now seeing increased interest due to their health benefits. These wheats differ from common and durum wheat in that their grains are covered by glumes, which are not removed during threshing (Shewry & Hey, 2015). Wheat is the primary source of carbohydrates in many countries and is the leading source of plant-based protein globally, with an average protein content of about 13%, which is relatively high for cereals. Whole grain wheat is also rich in micronutrients, dietary fiber, minerals, and vitamins, and it can provide a

balanced, nutritious diet when combined with other proteins, like animal or legume sources (Lafiandra et al., 2014; Shewry & Hey, 2015).

A wheat-based diet offers higher fiber content compared to meat-based diets. Many health claims approved by the European Food Safety Authority (EFSA) are linked to the beneficial fiber content in wheat and barley, particularly their role in supporting intestinal function, regulating glucose levels, and managing cholesterol. The most comprehensive research on wheat's nutritional value was conducted under the EU's HEALTHGRAIN program (*Healthgrain Project – Healthgrain Forum*, 2024). Wheat dough is unique due to its gluten content, which imparts viscoelastic properties essential for producing raised bread. Gluten proteins trap carbon dioxide bubbles during dough fermentation, causing it to rise (Graybosch, 1998). However, gluten is also associated with celiac disease, a chronic inflammatory condition that affects nutrient absorption and impacts about 1% of the population in Western Europe (Tye-Din et al., 2018). Wheat-related allergies, both respiratory and food-based, have spurred research into the health implications of wheat consumption. Globally, wheat consumption is increasing, even in regions with unfavorable climates for wheat production. In addition to being a dietary staple, wheat is commonly used as animal feed, especially when harvests are compromised, rendering some grains unsuitable for human consumption. This lower-quality wheat also finds industrial uses, such as in the production of adhesives, paper additives, and even alcohol (Johnson V. A. et al., 1978).

Barley (*Hordeum vulgare ssp. vulgare* L., 2n = 14, diploid, HH genomes) is one of the earliest domesticated crops, cultivated in Egypt's Nile River Valley around 17,000 years ago (Purugganan & Fuller, 2009; Wendorf et al., 1979). A post-domestication mutation leading to naked caryopsis, believed to have originated in the Middle East around 8000 BC, gradually spread, with evidence of hull-less barley found in Northern Scotland (Helbaek et al., 1969; Pourkheirandish & Komatsuda, 2007). In ancient Rome, gladiators were called "hordearii" (barley men) due to barley's significance in their diet. Over time, wheat replaced barley as the primary cereal, largely because it produced more grains per ear and was easier to thresh (http://www.barleyhub.org/). Today, barley ranks as the fourth most important cereal globally, grown in over 100 countries. Europe has contributed 60% of global production in the past decade, followed by Asia (15%) and the Americas (13%) (http://www.fao.org/ faostat/en/). Barley adapts

well to harsh conditions, tolerating cold, drought, and poor soil better than wheat (Kosová et al., 2014). Its primary use is for animal feed, but malting barley, used in beer and whisky production, is more valuable. Evidence of beer-making with barley dates back over 9000 years, with large-scale brewing emerging in pre-Dynastic Egypt 5000 to 7000 years ago. By 2014, global beer consumption reached over 1960 million hectolitres, requiring over 21.5 million tones of malt (http://e-malt.com/). Scottish distillers have also boosted malt whisky production during this time (http://www.ukmalt.com/malt-facts).

3.4 A brief history of hybridisation

The term "hybrid" likely originates from the Greek word "ὕβρις," meaning insult or outrage, particularly when directed toward the gods (hubris) or associated with sexual transgressions. Over time, the term evolved to mean "mongrel," possibly due to the belief that the creation of such creatures was a violation of nature. Well into the 18th century, hybridization was viewed with suspicion, and early plant breeders often felt the need to defend their work in crossing different species. There was a common belief that sexual relations between distinct species were unnatural and that creating new life forms was an affront to divine creation, implying a critique of the original act of Creation. The origin of "hybrid" thus initially referred to mixed-breed animals, but it was also applied to humans. For instance, a child born to a Roman father and an Asiatic or African mother, or a child from the union of a freeman and a slave, was called a hybrid (Zirkle, 1935)

The earliest recorded instance of creating an artificial hybrid was by London horticulturist Thomas Fairchild in 1717. He crossed *Dianthus barbatus* (♀) and *D. caryophyllus* (♂), producing offspring that displayed an intermediate phenotype, partially resembling both parent plants. Fairchild also observed that this plant hybrid, like the animal mule, was sterile and could only be propagated vegetatively (Durkin, 2024).

The first comprehensive scientific work on hybridization was published about half a century later by German botanist Joseph Gottlieb Koelreuter. Between 1760 and 1766, Koelreuter demonstrated the crossability of different species, based on 65 different crossing combinations across 13 genera and 54 species. His initial hybrid was a cross between *Nicotiana rustica* and *N.*

paniculata (Roberts, 1929*)* which proved to be partially fertile, unlike Fairchild's sterile hybrid. Koelreuter also made the important observation of frequent sterility in hybrid offspring, attributing this phenomenon to nature's way of preventing confusion from species interbreeding. He further documented the uniformity of progeny in crosses between species and genera, a discovery he validated by reciprocal crosses between *N. glutinosa* and *N. perennis* (Roberts 1929).

In cereals, the first recorded hybrid was produced by Wilson in 1876 through a cross between hexaploid wheat (*Triticum aestivum*) and rye (*Secale cereale*). The F1 plants he created were sterile, which is typical of hybrids (Bajaj, 1990; *Roberts_1929*, n.d.). However, the first fertile cereal hybrid is credited to German breeder Rimpau in 1891(Bajaj, 1990). He produced 12 fertile plants from the same wheat-rye combination, marking the first successful hybrid cereal, known as triticale. These plants closely resembled the maternal genotype and displayed a high degree of uniformity, producing consistent F2 generations with the same traits over many cycles of self-fertilization (Bajaj 1990). It wasn't until 45 years later that advance in cytology enabled Lindschau, Ohler, and Müntzing to confirm the hybrid's octoploid chromosome count - 56 chromosomes, 42 from wheat and 14 from rye - making it a new synthetic plant species (Bajaj, 1990; Lindschatt & OchIer, n.d.; Müntzing, 1936).

Following these early achievements, hybridization research expanded globally. In the latter half of the 20th century, as heterosis breeding gained prominence, there was a growing focus on creating hybrids by crossing plants with diverse traits (Bajaj, 1990). Hormone treatments and advanced tissue culture techniques made it possible to generate hybrid offspring from species combinations that were previously unimaginable (Molnár-Láng et al., 2011*).* These hybrids not only combined the beneficial traits of their parents but also exhibited new characteristics not found in either parent, such as novel flower shapes, sizes, and pigment variations resulting from enzyme interactions (Stebbins & Pun, 1953).

3.5 Advances in molecular cytogenetics and their impact on alien hybridization

At the end of the 20th century, significant advancements in molecular cytogenetics greatly advanced research into the creation of alien hybrids. In the 1990s, new technologies emerged that enabled the identification and tracking of DNA fragments from different parents. Hybridization techniques utilizing fluorescently labelled DNA probes (FISH) allowed for a more precise molecular cytogenetic analysis of cereal genomes than was previously possible (Jiang & Gill, 1994). The DNA assay, containing a high number of GAA trinucleotide repeats, produces a distinct hybridization pattern on cereal chromosomes, enabling clear differentiation between them (Pedersen et al., 1996). This method allows for the unique identification of chromosomes across various cereals, including those with complex genomes such as cultivated wheat (Pedersen & Langridge, 1997). It has also been highly effective in identifying chromosomes from different parents in hybrids and their derivatives resulting from interspecies crosses (Szakács & Molnár-Láng, 2007).

For hybrids of alien species, genetic material from the parents - whether whole genomes, entire chromosomes, or just chromosome fragments - can be separated through genome *in situ* hybridization (GISH) on mitotic or meiotic preparations of the offspring (Schwarzacher et al., 1989). This process uses fluorochrome-labelled DNA from the donor species as the hybridization probe, while unlabelled DNA from the recipient species serves as the blocking DNA. On labelled nuclear preparations, DNA fragments from each parent are clearly delineated, enabling the detection of intergenomic translocations. Moreover, the size of the integrated chromosomal segment and the position of the translocation breakpoint can also be determined (Mukai et al., 1993). The efficiency of the GISH method decreases with closer genetic relationships, making it less effective for closely related species, but it has proven highly successful for distant combinations like wheat × barley (Molnár-Láng et al., 2014).

By the late 1990s, advancements in molecular marker techniques further enhanced the ability to identify foreign chromosomes or chromosome segments incorporated into the genetic

material of offspring from alien crosses. These molecular markers, alongside cytological methods, provided highly accurate identification (Fedak, 1980). Currently, more than 2000 microsatellite markers have been mapped across the wheat genome, with similar numbers for rye (Korzun et al., 2001) and barley (Ramsay et al., 2000), facilitating the detection and delimitation of translocations between these species. For instance, the 1RS rye chromosome translocation (1BL-1RS) found in most commonly cultivated wheat varieties can be reliably detected using molecular markers. Additionally, the method has proven highly effective for molecular karyotyping of wheat × barley F1 hybrids (Hsam et al., 2000).

3.6 The genetic origins and crossability of cultivated wheat

Cultivated wheat (*Triticum aestivum*) is an allohexaploid species (2n = 6x = 42, AABBDD genome) that emerged from a natural, two-step hybridization involving three distinct diploid ancestors (2n = 2x = 14) approximately 8,000 to 10,000 years ago (Huang et al., 2002). This species is considered a true amphiploid, as it retains the full chromosome set from each of its progenitors. The three ancestors are identified as *Triticum urartu* for the A genome (Dvořák et al., 1993), *Aegilops speltoides* or a closely related species for the B genome (Sarkar & Stebbins, 1956; Tsunewaki, 2009), and *Aegilops tauschii* for the D genome (Mcfadden & Sears, 1946). These species belong to the *Triticeae* tribe, including both *Triticum* and *Aegilops* genera. The polyploid nature of wheat has resulted in intergenomic interactions that have facilitated the development of traits like fertility, baking quality, stem strength, and winter hardiness (Feldman & Levy, 2012). While the fusion of three genomes in wheat provides evolutionary advantages, it also poses significant challenges, such as limiting the number of wild species with which wheat can naturally crossbreed. The divergence of parental genomes during meiosis can lead to cytogenetic abnormalities, reducing the fertility and viability of offspring, and thus isolating the species from its wild relatives (Feldman & Levy, 2012; Kinoshita, 2007; Soltis & Soltis, 1999). Although most wheat-related species can cross with wheat, the resulting hybrids often have low fertility, making the maintenance of these hybrids challenging (Belea 1976). Since there are no naturally occurring species carrying only the AABBDD genome techniques such as assisted pollination, hormone

treatments, and *in vitro* methods are necessary to enable gene transfer across species boundaries (Peng et al., 2011).

3.7 Genetic resources of wheat

Wheat gene source species can be categorized into three groups based on their genome composition (Friebe et al., 1996). The primary gene sources include hexaploid wheat landraces, cultivated tetraploid *durum* (2n = 4x= 28, AABB) wild *T. diccoides*, and the diploid donors of A and D genomes to *durum* and bread wheat (Friebe et al., 1996). However, because unpaired chromosomes are unstable during the meiosis of the F1 generation, multiple backcrosses are necessary to stabilize the introduced traits (Thompson, 1930).

Secondary gene sources include species that share at least one genome homologous to hexaploid wheat, such as polyploid species of *Aegilops* and *Triticum* (Friebe et al., 1996). Due to genome incompatibility, these species often require *in vitro* embryo culture to produce viable hybrids (Molnár-Láng et al., 2011). Although germination rates in such combinations are typically high, the resulting F1 hybrids are usually sterile, and obtaining viable seed crops naturally is rare (Thompson, 1930). This sterility arises from genetic incompatibility, where non-homologous genomes act partially or entirely haploid during meiosis, leading to uneven distribution of chromosomes in gametes and reduced fertility. Fertility can sometimes be restored through multiple backcrossing or whole genome duplication (Molnár-Láng et al., 2011).

Tertiary gene sources encompass species that do not share any genomes with hexaploid wheat, such as *Secale*, *Hordeum*, and *Agropyron*. Although these species show significant genetic diversity, they can still produce viable offspring, albeit in small numbers (Friebe et al., 1996). Cultivation of these hybrids almost always requires *in vitro* embryo culture, and genome duplication is necessary to restore fertility in the F1 progeny due to haploid-like behaviour during meiosis (Molnár-Láng et al., 2011).

3.8 The role of alien crosses in breeding

Wild relatives of hexaploid wheat possess numerous beneficial agronomic traits, including resistance to various abiotic and biotic stresses (Molnár-Láng et al., 2011). These wild species can be crossed with wheat, allowing them to transfer their advantageous traits to hybrid offspring (Belea, 1976). While the process of producing such hybrids is relatively straightforward, it is important to recognize that genes responsible for desirable traits may be linked to DNA segments associated with less favourable agronomic characteristics (Ortelli et al., 1996).

The primary aim of incorporating genes from alien species is to integrate the shortest possible DNA fragment that solely carries advantageous agronomic traits into the wheat genome through translocations. Achieving this separation of beneficial genes from undesirable ones requires extensive effort, including multiple rounds of backcrossing and selection (Molnár-Láng et al., 2011). To streamline this process, it is advisable to select relatives that carry the gene for the desired trait but have had the genes linked to unfavourable traits removed during domestication (Kole, 2011).

Fortunately, the grass family is rich in domesticated species (Kole, 2011), though none of the commonly cultivated species are directly related to hexaploid wheat, meaning they do not fall under its primary gene sources (Friebe et al., 1996). Nonetheless, certain domesticated relatives of wheat can be utilized for sexual intercrossing and may possess valuable agronomic traits that are absent in cultivated wheat (Kole, 2011).

3.9 Rye and barley as potential crossbreeding partners

Among cultivated cereals, rye (*Secale cereale*) (2n = 2x = 14, RR genome) is the species most closely related to wheat (Bendich & McCarthy, 1970a). Rye is notable for its high adaptability, excelling in cold and drought tolerance, with better resistance to diseases like leaf rust and powdery mildew compared to wheat, and being less demanding in terms of soil and growing conditions (Kole, 2011). It can be crossed with wheat, producing partially fertile F1 hybrids that exhibit intermediate characteristics of both parents (Hsam et al., 2000; Lindschau and Ohler, 1935; Belea,

1976). This hybridization has led to the development of an amphiploid between hexaploid *T. aestivum* wheat and rye, and also to the creation of hexaploid *triticale* from tetraploid wheat (*T. durum*) and rye. *Triticale*, the first synthetic amphiploid plant, has gained significant economic importance due to its advantageous traits. Another key result of wheat-rye hybridization is the 1B-1R translocation, which is now present in many widely grown wheat varieties (Hsam et al., 2000).

Barley (*Hordeum vulgare*, 2n = 2x = 14, HH genome) is a more distant relative of wheat than rye, as indicated by genome analysis (Bendich & McCarthy, 1970a; Moore et al., 1993). It is also categorized as a tertiary gene source for wheat, with the two species believed to have diverged in the Pleistocene era (Friebe et al., 1996; Moore et al., 1993). Barley is still the most important cereal in the cold temperate regions and has valuable traits distinct from wheat. It is one of the oldest domesticated crops, and winter barley is harvested earlier than winter wheat. Some barley genotypes are highly tolerant to salt and drought, and barley also contains higher levels of key amino acids, such as lysine, compared to wheat, crossing barley and wheat, two of Europe's most significant cereal crops, offers the potential to merge beneficial traits developed through their separate domestication histories (Molnár-Láng et al., 2011).

Although barley and rye can be crossed, attempts at hybridization have so far resulted in haploid offspring with only the maternal genome, as the paternal genome is lost early in embryo development (Sharma, 1995). Crosses between tetraploid barley and diploid rye have similarly produced diploid barley offspring (Bajaj et al., 1986).

3.10 Wheat (*T. aestivum*) × Barley (*H. vulgare*) hybrids

Crosses between species from the genera *Triticum* and *Hordeum* have already resulted in the creation of fertile amphiploids, termed tritordeum, similarly named like *triticale*. The octoploid *tritordeum* (2n = 8x = 56) originated from crosses between *Hordeum chilense* and *T. aestivum*, while the hexaploid tritordeum (2n = 6x = 42) was developed from crosses between *H. chilense* and *T. turgidum*, with genome duplication in F1 hybrids (Martin & Chapman, 1977). Despite the useful agronomic traits displayed by these synthetic amphiploids (Hernández et al., 2001; Martin

& Cubero, 1981) their adoption has been limited, largely due to the undesirable characteristics of wild *H. chilense* (Hernández et al., 1999).

The first recorded attempt to cross the cultivated species *T. aestivum* and *H. vulgare* occurred in the early 1900s (Farrer, 1904). However, this, like many subsequent efforts, was unsuccessful. Despite many decades of experimentation, a stable amphiploid hybrid has yet to be produced. One of the first successful crosses was by Kruse (1973), who used four-row barley (*H. vulgare*) as the female parent and various wheat species (*T. aestivum, T. dicoccum, T. monococcum*) as male parents. By employing *in vitro* embryo culture, a viable plant was obtained (Kruse, 1973) although the hybrid nature of the progeny was not definitively proven (Bajaj, 1990). Since then, viable partial hybrids have been generated using a range of cross combinations (Islam et al., 1975; Fedak, 1980; Islam et al., 1981; Mujeeb-Kazi & Rodriguez, 1984; Molnár-Láng et al., 1985; Bajaj, 1990; Koba and Shimada, 1992; Polgári et al., 2014). In general, results have shown that when barley is the female parent, the success rate is higher, with a grain set rate of 15.4% (Islam et al., 1975) compared to 1.3% in the reverse cross (Islam et al., 1981). However, progeny from these crosses were male sterile, likely due to a cytoplasmic inheritance factor, and both F1 and backcross (BC) populations displayed pistilloidy, where flowers took on fruit-like forms (Islam et al., 1981; Mujeeb-Kazi and Rodriguez, 1984).

When wheat is the female parent, fertile offspring have occasionally been produced in both F1 and BC populations, which have been self-fertilized to form addition lines incorporating some barley chromosomes (Islam et al., 1981). Initial successes led to widespread research aimed at creating lines combining wheat and barley genomes. This work produced lines like Mv9Kr1 × 'Igri', which displayed valuable agronomic traits (Szakács & Molnár-Láng, 2007), though a fully stable wheat-barley amphiploid, similar to triticale, has yet to be created. Hormone treatment has been shown to improve embryo yield. Koba and colleagues, for example, achieved an 8.3% embryo yield by using 2,4-D, a method previously proven in wheat × maize crosses, and successfully regenerated plants through embryo culture (Koba & Shimada, 1992). Most plants produced through these methods contained portions of the barley genome but showed significant chromosomal mosaicism (Koba & Shimada, 1992). Tri-lineage hybrids have also been created by combining *H. vulgare*, *T. aestivum, and S. cereale, or S. montanum* (Bajaj, 1990), although these

hybrids exhibited high chromosomal instability due to the interactions of genomes from different species (Bajaj, 1990).

Only a few studies have explored the reasons behind the near-complete incompatibility between wheat and barley, and the exact causes remain unclear. Some studies have linked it to abnormalities in pollen development (Vishnyakova & Willemse, 1994), while others found no connection between seed set and pollen germination activity, pollen tube growth rates, or pollen grain abnormalities. In some instances, barley pollen germinated on wheat stigmas more frequently and faster than wheat's own pollen, with no noticeable difference in the appearance of the pollen tubes (Neeraj and Khanna, 1992).

3.11 Detection and characterization of alien chromatin or chromosomes transfer in wheat x barley hybrid

Bread wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) rank among the most critical cereal crops globally. Hybridization between these species enables the transfer of desirable traits, such as earliness, from barley into wheat (Kruse, 1973). Introgressive hybridization, one of the most effective plant breeding strategies, introduces beneficial genes from wild or alien species into cultivated crops. Hybridization with an alien species or genus is commonly followed by allopolyploid production and backcross breeding to incorporate alien genes into crop species. Methods such as manipulating homoeologous chromosome pairing, ionizing radiation, using gametocidal chromosomes, tissue culture, centric breakage, and fusion allow the development of recombinant lines containing the desired alien genes. This introgression process can occur through chromosome addition, substitution lines, or spontaneous and/or induced translocations (Banks et al., 1995).

Economically valuable traits have been successfully integrated into wheat as single genes, chromosomal arms, or entire chromosomes. Genomic *In situ* hybridization (GISH) stands as the most efficient technique for locating breakpoints and estimating alien chromatin quantity within translocated chromosomes (Le et al., 1989; Schwarzacher et al., 1989; Jiang & Gill, 1994b). Accurately detecting foreign chromatin in recipient progenies significantly supports successful

gene transfers. Chromosome engineering, identification, and characterization are essential for genetic manipulation and gene transfer in wheat breeding (Mascher et al., 2017).

Previously, meiotic analyses were the sole means of identifying translocated chromosomes, later complemented by chromosomal banding. More recently, *In situ* hybridization (ISH), utilizing either whole-genome DNA probes or genome-specific repetitive DNA probes, has advanced these analyses. Techniques such as GISH, alone or combined with C-banding and/or fluorescence *In situ* hybridization (FISH), have been instrumental in identifying, locating, and measuring the introgressed alien chromatin, translocation breakpoints, and tracking alien chromosome segments throughout backcrossing and selection. GISH's capacity to detect minor DNA introgressions is especially valuable. For introgressive hybridization to succeed, stable incorporation and transmission of alien chromatin into progeny over successive generations are critical. GISH has thus been invaluable for monitoring foreign chromatin across generations, especially when conventional cytogenetic analysis is limited (Mascher et al., 2017).

3.12 *In situ* hybridizations techniques

In situ hybridization (ISH) is a high-resolution cytogenetic technique that allows for the detection, quantification, and localization of nucleic acid targets within cells or tissues. It relies on sequence-specific complementary probes, usually DNA, that hybridize with their target sequences in the cell nucleus, allowing for the visualization of DNA or RNA within chromosomes or cellular structures. ISH can use radioactively or fluorescently labelled probes or indirect methods like histochemical markers, enabling the study of gene expression and chromosomal structures in individual cells. *In situ* hybridization (ISH) evolved later than immunofluorescence-based methods for protein detection. Immunofluorescence, pioneered by Coons et al. in 1941 and widely applied by the 1950s, laid the groundwork for visualizing cellular components with fluorescent tagging (Coons et al., 1941). About three decades later, ISH was introduced, marking a milestone in cellular and molecular biology. The foundational principles of DNA melting, re-hybridization, and RNA-DNA hybridization were described in the 1960s, setting the stage for ISH development. In 1969, Pardue and Gall made a significant breakthrough by tagging ribosomal RNA (rRNA) probes with H3 to visualize rRNA-encoding genes auto radiographically in *Xenopus laevis* oocytes(Pardue & Gall,

1969). Parallel work by John et al. and Buongiorno-Nardelli with Amaldi further advanced ISH techniques for detecting rDNA in Xenopus and paraffin-embedded tissues (Buongiorno-Nardelli & Amaldi, 1970; John et al., 1969).

In the following years, Gall and colleagues expanded ISH use in mammalian cells, visualizing satellite DNA in the heterochromatic regions of mouse chromosomes (Pardue & Gall, 1970). Early ISH applications were, however, limited by low sensitivity and scarce sequence-specific probes. Although radioisotope labeling provided high sensitivity, it also required long exposure times (up to weeks for H3 detection), suffered from low spatial resolution (in megabases), and presented high background noise. These constraints catalyzed the development of non-isotopic probe labeling methods (Pardue & Gall, 1969).

The progression of ISH into fluorescence *In situ* hybridization (FISH) in the 1970s allowed for significant advancements in cellular imaging and probe specificity. By the 1990s, direct probe labeling and refined FISH probe designs had enhanced resolution and background reduction, making FISH a powerful tool for cytogenetics and molecular biology (Gillespie & Spiegelman, 1965; Watson & Crick, 1953). The integration of microfluidics with FISH in the early 21st century represents a more recent advancement, enabling precise and efficient analysis at the single-cell level (Chen et al., 2015).

3.13 Fluorescence *In situ* hybridization (FISH)

The initial molecular cytogenetic techniques used radioactively labelled probes. The isotope tritium [^{3}H] is largely used because of its low energy radiation, which guarantees better probe resolution. Other isotopes are also used, such as [^{125}I], [^{35}S], and [^{32}P]. In general, the radioactive isotope is chosen according to the resolution level desired. Currently, radioactive probes are rarely used, as they demand a long exposure time and also endanger the health of those who handle them. Nowadays, non-radioactive probes are used, where a label is bound to the DNA probe. The most used labeling molecules are digoxigenin and biotin, which are detected by means of fluorochromes (direct staining) or by an antibody-fluorochrome conjugate (indirect staining). With the introduction of fluorochromes, this technique became known as Fluorescent *In Situ* Hybridization

(FISH) (Figure 3) (Guerra, 2004). This technique is used for the spatial detection and quantification of nucleic acids in their cellular environment (Huber, 2018). Chromosomal physical mapping and the study of evolutionary chromosome rearrangements can both be accomplished with the aid of (FISH). Individual wheat chromosomes and chromosomes from related species can be identified using tandem repeats and microsatellites labelled with FISH (Danilova et al., 2014). It enables the concurrent viewing of many DNA targets using the same specimen, on the same sample, is an effective and highly reproducible method to examine fundamental features of chromosomal behavior during meiosis, like pairing between species. FISH can not only supplement the information provided by other cytogenetic techniques, both conventional and molecular, but also increase the scope of information possible for theoretical and applied study, especially when there is little existing knowledge on the genetic or structural similarities between the alien and chromosomes from wheat (Cuadrado et al., 1997). An essential stage in the investigation of genetic interactions is chromosomal identification. FISH is useful in this process because it can swiftly and precisely identify chromosomes. More plant chromosomes can now be distinguished by FISH due to the introduction of FISH probes, including those in Arabidopsis, wheat, barley, maize, soybean (Han et al., 2019).

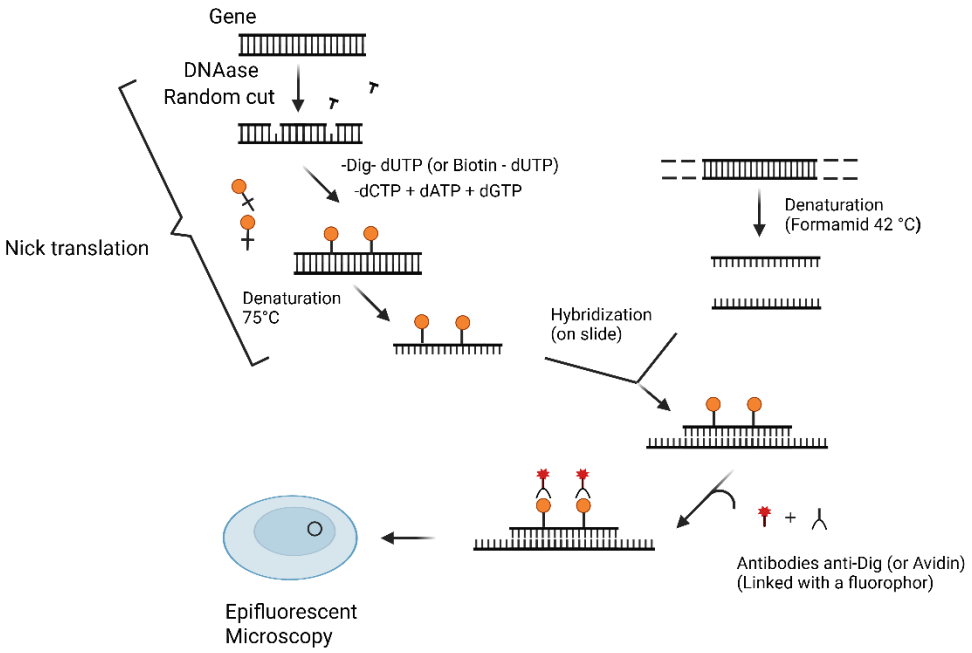

Figure 3: The image illustrates the Fluorescence *In Situ* Hybridization (FISH) process. The process begins with nick translation, where DN-ase creates random cuts in the DNA, and labelled nucleotides (Dig-dUTP or Biotin-dUTP) are incorporated during synthesis alongside dCTP, dATP, and dGTP. The labelled DNA is denatured at 75°C and hybridized with denatured cellular DNA on fixed slides after treatment with formamide at 42°C. During hybridization, the labelled probe binds to the target sequence, and detection occurs through antibodies (anti-Dig) or Avidin linked with a fluorophore. Finally, epifluorescent microscopy is used to visualize the fluorescent signal, pinpointing the gene's location within the cell nucleus (Image created in Bio render).

3.14 Genome *In situ* hybridization (GISH)

One of the most fascinating and functional research techniques to emerge just a little more than two decades ago is genomic *In situ* hybridization, a modification of fluorescence *In situ* hybridization technology. It has made it possible for plant chromosomal and genome research to advance at an incredible rate, and its potential is yet very much untapped. GISH has been used to distinguish chromosomes from different parents or genomes within interspecific, intergeneric hybrids, or allopolyploids. In GISH, total genomic DNA from one parent is labelled as a probe, while DNA from the other parent is added in higher concentrations as a blocking DNA. This blocking DNA binds to shared sequences, preventing cross-hybridization and enhancing

specificity (Peñaloza and Pozzobon, 2007). For GISH, genomic DNA is fragmented using restriction enzymes, autoclaving, or sonication, with sonication offering more precision. Probe labeling typically uses kits for nick translation (Figure 4) (Xiong et al., 2006).

GISH has been highly effective in plant research, particularly in developing interspecific hybrids for crop improvement, such as pathogen resistance or yield increases. In rice (*Oryza*), GISH has been used to differentiate genomes in hybrids, like those between *O. sativa* and *O. meyeriana*, showing distinct genome features due to heterochromatin content (Xiong et al., 2006). However, hybrids between closely related species, such as *O. sativa* and *O. rufipogon*, are challenging to distinguish without high blocking DNA concentrations due to their genetic similarity, suggesting a close evolutionary relationship (Tan et al., 2006). GISH has also identified genome structures in other genera, such as *Setaria*, where it revealed that *S. queenslandica* is an autotetraploid with an AAAA genome configuration by staining all chromosomes with the probe from *S. viridis* but none with *S. adhaerens* (Wang et al., 2009). Additionally, GISH confirmed that *Clivia cyrtanthiflora*, an ornamental hybrid developed by Charles Raes, is an F1 cross between *C. miniate* and *C. nobilis* (Ran et al., 2001).

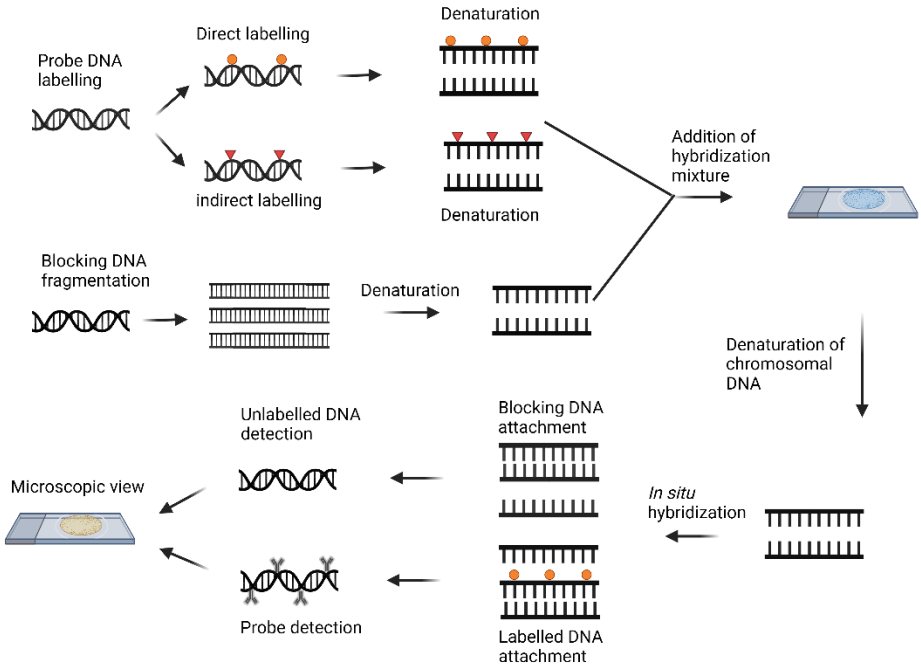

Figure 4: This diagram illustrates the process of Genomic *In Situ* Hybridization (GISH), a technique used to identify and visualize specific DNA sequences from different genomes within hybrid plants. The process begins with probe labeling, where genomic DNA from one of the parental species is labelled with a fluorochrome either directly or indirectly. Fragmentation and denaturation of blocking DNA prepare unlabelled genomic DNA to prevent non-specific binding and increase the specificity of hybridization. Slide preparation involves placing chromosome spreads on a slide, followed by probe denaturation to ensure the probe DNA is single-stranded. The hybridization mixture, which combines the labelled probe with blocking DNA, is then added. Denaturation of chromosome DNA on the slide makes it single-stranded and ready for hybridization, allowing the labelled DNA probe to bind to its complementary sequences during in situ hybridization. Probe detection involves using antibodies associated with fluorochromes to visualize the hybridized probe. Finally, visualization under a microscope enables the identification of specific genomes within the hybrid chromosomes, helping to distinguish between parental DNA contributions (Image created in Bio render).

GISH has been instrumental in identifying and analyzing chromosomes in intergeneric hybrids. First applied by (Schwarzacher et al., 1989). GISH demonstrated clear identification of chromosomes in hybrids such as those between *Hordeum chilense* and *Secale africanum*, revealing distinct chromosomal domains that affect cellular functions like gene expression. The technique has since enabled chromosome distinction in hybrids across various genera. In somatic hybrids of *Citrus auratium* and *Poncirus trifoliata*, GISH identified chromosomes from each species and

detected recombinant chromosomes (Fu et al., 2004). For *Brassicaceae*, GISH, combined with cytogenetics, accurately identified chromosome origins in hybrids like *Brassicoraphanus*, a cross of *Brassica campestris* and *Raphanus sativus* (Lim et al., 2012).

GISH has also been applied to study hybridization among *Littonia*, *Sandersonia*, and *Gloriosa* species, revealing distinct genomes and limited cross-hybridization, suggesting early divergence among these genera (Nakazawa et al., 2011). Similarly, GISH distinguished chromosomes in hybrids between *D. nankingense* and *T. vulgare*, indicating a distant relationship due to the absence of blocking DNA requirements (Tang et al., 2011). In *Lycopersicon esculentum* and *S. lycopersicoides* hybrids, GISH identified specific chromosome sets in tetraploid and hexaploid hybrids, with variations that correlated to morphological differences, such as leaf shape. Through these studies, GISH has provided insights into genetic relationships, chromosome organization, and evolutionary divergence in plant species (Escalante et al., 1998).

3.15 Limitations of GISH

Genomic *In Situ* Hybridization (GISH) has become invaluable in plant cytogenetics, particularly for studying hybrids. Traditionally, characterizing wheat introgressions from alien species involved analyzing meiotic metaphase-I pairing with testers, a time-intensive and imprecise technique (Sears, 1978). However, GISH and DNA marker mapping revealed limitations in this older method, showing translocation breakpoints could sometimes be misjudged or misassigned to incorrect chromosomes or arms (Ceoloni et al., 1996; Eizenga, 1987; H.-B. Zhang & Dvořák, 1990). GISH, using either enzymatic color reactions (Rayburn and Gill, 1985) or fluorescent labells (Schwarzacher et al., 1989), has since provided direct, reliable physical mapping and has become the preferred approach for characterizing known translocations (Friebe, 1996). However, GISH has been less frequently applied for discovering new introgressions due to its cost and technical demands (Masoudi-Nejad et al., 2002).

GISH faces limitations in resolution, particularly near telomeres, where it may fail to detect small chromosome segments from non-probe species due to probe halo effects. This issue is significant when analyzing distal translocation breakpoints, as it can hinder detection of some

recombinant events, thus limiting the genetic variation available for developing alien introgressions (Lukaszewski, 1995; Lukaszewski & Curtis, 1993). Recently, Dr. T.R. Endo's lab at Kyoto University simplified GISH for cost-effective screening of large populations. This refinement in GISH application highlights the importance of precision in identifying translocated chromosomes and underscores ongoing challenges in resolution, particularly with distal recombination events in wheat-rye hybrids and other recombinant chromosomal studies involving *Agropyron elongatum* (Masoudi-Nejad et al., 2002).

3. 16 Advancements in genome editing

The development of plants with enhanced traits is essential for modern agriculture and various industries that rely on plant resources. Traditionally, plant breeding has been achieved through crossing and selection, but these methods are time-consuming and labor-intensive. Genome editing offers a precise approach to modify specific DNA sequences, significantly improving efficiency (Osakabe & Osakabe, 2015; Wang et al., 2016; Jaganathan et al., 2018). The process of genome editing typically involves three key steps. First, an engineered nuclease with a recognition module and a nuclease domain identifies the target DNA sequence. Once bound, the nuclease induces double-strand breaks (DSBs) at or near the target site. These breaks are then repaired through endogenous pathways, either nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) (Osakabe & Osakabe, 2015; Jaganathan et al., 2018). NHEJ is prone to errors and often introduces small insertions or deletions (Indels), whereas HDR enables precise DNA repair. These genome-editing technologies have been successfully utilized in a variety of organisms, including plants (Osakabe & Osakabe, 2015; Wang et al., 2016; Jaganathan et al., 2018).

There are three primary genome-editing technologies. Zinc finger nucleases (ZFNs) were the first reported engineered nucleases, followed by transcription activator-like effector nucleases (TALENs), which offered greater flexibility. More recently, the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system has emerged as a simpler and more adaptable genome-editing tool. Both ZFNs and TALENs consist of a sequence-specific DNA-binding module linked to a FokI nuclease domain, which requires dimerization for cutting both DNA-strands. This means two separate components must be designed to target closely

spaced DNA sequences, ensuring specificity but also increasing complexity and cost (Figure 5 A and B). In contrast, the CRISPR/Cas9 system is more cost-effective and easier to design (Osakabe & Osakabe, 2015; Wang et al., 2016; Jaganathan et al., 2018). Introduced as a genome-editing tool in 2012, CRISPR/Cas9 has rapidly become the most widely applied method due to its efficiency, simplicity, and versatility (Jinek et al., 2012; Cong et al., 2013; Wada et al., 2020a). It consists of two main components: the Cas9 protein, an RNA-guided DNA endonuclease, and a guide RNA (gRNA) that directs Cas9 to the target site (Figure 5C). Unlike ZFNs and TALENs, which rely on DNA-protein interactions for specificity, CRISPR/Cas9 uses DNA-RNA base pairing, simplifying the design process. While ZFNs and TALENs require the development of distinct DNA-binding domains (500–700 amino acids in TALENs) for each target, CRISPR/Cas9 only requires designing a short 18–20 base pair oligonucleotide, making it a more accessible genome-editing approach. For CRISPR/Cas9 to function, Cas9 and gRNA must recognize and bind to a protospacer adjacent motif (PAM), a short nucleotide sequence located at the 3′ end of the target sequence. The most commonly used Cas9 variant, derived from *Streptococcus pyogenes* (SpCas9), recognizes the PAM sequence 5′-NGG-3′. Once recruited, Cas9 induces DSBs at the target site, though unintended off-target effects can sometimes occur. To improve precision and minimize genomic disruptions, advancements have been made to enhance the specificity of CRISPR/Cas9 or avoid DSBs altogether (Osakabe & Osakabe, 2015; Wang et al., 2016). Additionally, careful strategies are employed to remove exogenous transgenes when developing desired mutant plants (Wang et al., 2016).

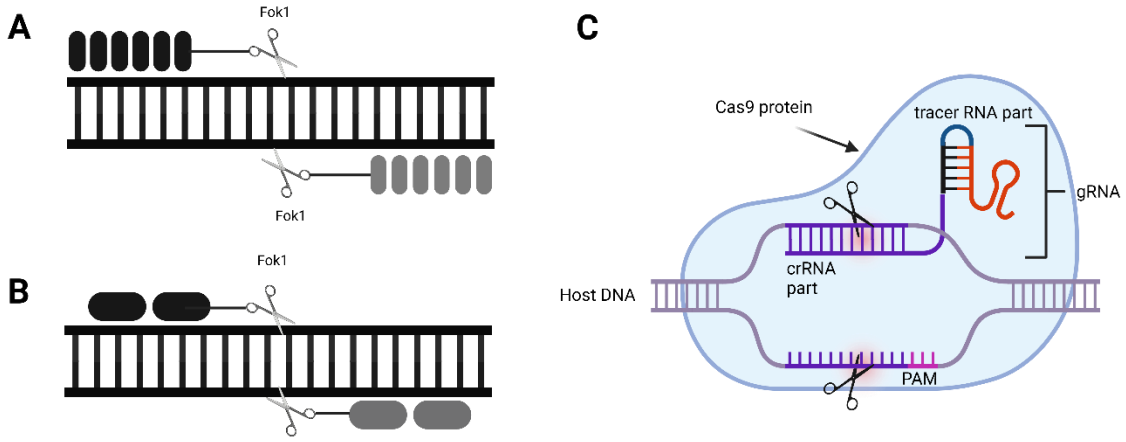

Figure 5: Image showing three mostly utilized targeted genome editing tools: Transcription Activator-Like Effector Nucleases (TALENs), Zinc Finger Nucleases (ZFNs), and CRISPR-Cas9. **A:** TALENs use TALE protein domains (each recognizing a single nucleotide) for specific DNA binding, fused to a FokI nuclease that dimerizes to induce double-strand breaks. **B:** ZFNs use engineered zinc finger domains (each recognizing 3 base pairs) to target DNA sequences, also fused to a FokI nuclease for DNA cleavage. **C:** CRISPR-Cas9 employs a guide RNA (consisting of a crRNA and a tracrRNA or a combined guide RNA) to direct the Cas9 protein to a complementary DNA sequence next to a PAM motif, where Cas9 induces a double-strand break (Image created in Bio render).

3.17 Genome editing using CRISPR/Cas9 in plants

The CRISPR/Cas9 system has been effectively utilized across various plant species, including both model organisms like *Arabidopsis* and economically significant crops such as rice, tobacco, sorghum, wheat, maize, soybean, tomato, potato, poplar, apple, and banana (Osakabe & Osakabe, 2015; Wang et al., 2016; Jaganathan et al., 2018). Different plant tissues, including calli, embryo, leaf discs, protoplasts, and flowers, have served as starting materials for genome editing. The objectives of these applications range from improving resistance to abiotic and biotic stresses to modifying metabolic pathways and increasing grain yield. Importantly, mutations introduced through CRISPR/Cas9 are heritable, demonstrating its potential for both plant research and agricultural advancements. A key advantage of the CRISPR/Cas9 system is its ability to edit multiple genes simultaneously (Zsögön et al., 2018; Armario Najera et al., 2019). In addition to modifying multiple genes, CRISPR/Cas9-mediated editing can induce targeted deletions of

specific DNA segments between editing sites. This capability is valuable for disrupting regulatory sequences and generating knockout mutants by deleting entire genomic regions rather than relying on frame-shift mutations. Another precise genome-editing strategy, known as gene targeting (GT), employs CRISPR/Cas9 to introduce specific changes via the homology-directed repair (HDR) pathway. However, HDR efficiency in plant cells is significantly lower than that of nonhomologous end-joining (NHEJ), prompting ongoing research into methods to enhance GT effectiveness (Armario Najera et al., 2019).

Despite the advantages of CRISPR/Cas9, a major concern is the potential for off-target effects - unintended genetic modifications occurring at non-target sites. Various methods have been developed to detect such mutations both *in vitro* and *in vivo*, including SITE-seq (Cameron et al., 2017), Digenome-seq (Kim et al., 2015), CIRCLE-seq (Tsai et al., 2017), GUIDE-seq (Tsai et al., 2015), and DISCOVER-seq (Wienert et al., 2019). Additionally, efforts have been made to modify *Cas9* proteins to enhance target specificity. Another recently identified issue in genome editing involves unexpected mutations. Some reported occurrences of large deletions—up to 9.5 kb—in mammalian cells as a result of *Cas9*-mediated editing. While such large deletions have not yet been documented in plants, their potential presence should be considered in future research to ensure the precision and safety of genome-editing applications (Kosicki et al., 2018).

3.18 Advancements in transgene-free plant genome editing

The advent of CRISPR/Cas9-mediated genome editing has driven efforts to establish efficient methods for generating transgene-free genome-edited plants. Eliminating transgenes from genome-edited plants prevents unintended genomic modifications, reduces concerns regarding off-target effects, and helps to address regulatory and legal issues associated with genetically modified organisms (GMOs). Several strategies have been developed to achieve this goal, including Mendelian segregation, programmed self-elimination, transient expression, and ribonucleoprotein (RNP)-mediated genome editing (Wada et al., 2020b).

3.18.1 Mendelian Segregation

The broadest strategy to obtain transgene-free plants is Mendelian segregation-based. CRISPR/Cas9 constructs can be delivered as DNA, and primary transformants are detected using antibiotic resistance markers. When these transgenic plants are allowed to undergo sexual reproduction, the transgenes segregate in subsequent generations according to Mendelian inheritance. This allows the isolation of null segregants - offspring having stably lost the transgenic cassette but have retained the desired edits. To facilitate selection, visual markers (e.g., mCherry fluorescence) are now used to rapidly to identify transgene-free plants without antibiotic screening, saving labor and speeding the breeding process (Gao et al., 2016; Lu et al., 2017). However, studies have shown that remnants of transgenes, such as vector backbone sequences or small untraceable DNA fragments, may still integrate into the plant genome and persist undetected. This raises concerns about the complete elimination of foreign DNA and highlights the need for more precise genome-editing strategies (Kohli et al., 1998; Forsbach et al., 2003).

3.18.2 Programmed Self-Elimination of Transgenic Plants

Programmed self-elimination techniques have been investigated to accelerate the process of null segregant selection. He et al. (2018) developed a system in rice that couples the cytotoxic BARNASE gene with cytoplasmic male sterility (CMS) to selectively kill transgenic plants. This strategy utilizes BARNASE, which is under the control of a germination-specific promoter to terminate transgenic T1 seedlings, and CMS guarantees that the eggs are fertilized exclusively by non-transgenic pollen to yield 50% null segregants (transgene-free) in the T1 generation. The elimination of transgenic offspring enables natural enrichment of null segregants without chemical selection. This technique is based on sexual reproduction and therefore can be applied only to seed-propagated crops, thereby limiting its general applicability (He et al., 2018).

3.18.3 Transient Expression of CRISPR/Cas9

A promising alternative to avoid transgene integration is the transient expression of CRISPR/Cas9 via DNA or mRNA. Protoplast transformation has been successfully used in potato and other plant

species to achieve genome editing, though protoplast regeneration remains a challenge (Andersson et al., 2017; Lin et al., 2018). *Agrobacterium*-mediated transient expression has also been applied to isolate null segregants in tobacco. It is also demonstrated that *in vitro*-transcribed Cas9 mRNA could enhance genome editing efficiency in wheat without transgene integration (Zhang et al., 2016a; Chen et al., 2018).

3.18.4 RNP-Mediated Genome Editing

The use of RNP complexes, where preassembled Cas9 protein and gRNA are directly delivered into plant cells, offers a transgene-free genome-editing approach (Woo et al., 2015b). Subsequent studies applied RNP-based genome editing to grapevine, apple (Malnoy et al., 2016), wheat (Liang et al., 2017), cabbage, and Chinese cabbage (Murovec et al., 2018). The method has also been successfully implemented using biolistic transformation in maize (Svitashev et al., 2016) and wheat (Liang et al., 2017), achieving significant genome-editing efficiency with minimal off-target effects.

3. 19 Utilization of the CRISPR/Cas9 System for crop improvement

Improving crop yield and nutritional value is a fundamental aspect of crop enhancement strategies aimed at meeting future food requirements and promoting human health. The CRISPR-Cas9 system was used to edit drought-responsive transcription factor genes in wheat protoplasts, specifically targeting dehydration-responsive element binding protein 2 (*TaDREB2*) and ethylene response factor 3 (*TaERF3*), through transient expression of sgRNA and Cas9 protein. Similarly, CRISPR-mediated knockout studies confirmed the role of the *TaHAG1* gene in wheat salinity tolerance (Kim et al., 2018).

In another study, base-editing techniques were used to modify two key wheat genes - acetolactate synthase (*TaALS*) and acetyl-coenzyme A carboxylase (*TaACC*) - to create transgene-free mutants resistant to various commercial herbicides. The integration of these traits into wheat lines offers potential benefits for weed control during the cropping season (Zhang et al., 2019).

Additionally, CRISPR-based editing has targeted the *DIW1/TaPP2C158* gene, resulting in drought-tolerant wheat plants (Wang et al., 2023).

The CRISPR-Cas9 system has been used to develop disease-resistant wheat varieties by knocking out genes associated with pathogen sensitivity. For instance, a successful mutation in the *TaEDR1* gene - a negative regulator of powdery mildew (*Blumeria graminis f. sp. tritici)* resistance - can produce wheat plants with enhanced disease resistance (Zhang et al., 2016). Furthermore, mutations in all six *MLO* alleles led to the development of *mlo* triple mutants, which exhibited increased resistance to Powdery mildew, achieved through transient protoplast expression (Shan et al., 2014). Mutating a single TaMLO-A1 allele in the A genome was also shown to enhance resistance to Pm (Wang et al., 2014).

CRISPR-based knockout of the histidine-rich calcium-binding protein (*TaHRC*) gene in hexaploid bread wheat has also conferred resistance to Fusarium head blight (FHB). This gene is directly linked to QTL Fhb1, a determinant of FHB susceptibility, and its disruption renders the gene non-functional, thereby promoting FHB resistance (Su et al., 2019). Moreover, knocking down wheat calcineurin B-like protein (CBL)-interacting protein kinases (*TaCIPKs*), which are susceptibility factors for wheat stripe (yellow) rust fungal pathogen (Pst) infections, significantly improved disease resistance by increasing ROS accumulation and the expression of pathogenesis-related (PR) genes (He et al., 2023). Manipulation of genes related to salicylic acid (SA) and jasmonic acid (JA) biosynthesis through CRISPR has further demonstrated potential for enhancing wheat immunity against various pathogens (Mishra et al., 2024).

Yield improvement has also been achieved using CRISPR-based gene editing. The knockout of three homoeoalleles of the *TaGW2* gene led to increased thousand kernel weight (TKW) and larger seed size in wheat (Wang et al., 2018). Additionally, CRISPR-based editing of *TaNP1* homoeoalleles resulted in *TaNP1* triple mutants, which exhibited complete male sterility (Li et al., 2020). CRISPR-induced mutation in the centromeric histone *TaCENH3a* created paternal haploid inducer wheat lines, which promote fertilization and enhance crop productivity (Lv et al., 2020). Furthermore, the knockout of *TaPHO2-A1* in bread wheat improved phosphorus (Pi) acquisition and grain yield under low-phosphorus conditions without adverse effects in high-P

environments (Ouyang et al., 2016). These genetically edited wheat lines have the potential to be released as new varieties or used as germplasm for future breeding improvements (Hussain et al., 2022).

An innovative approach to increasing wheat yield through CRISPR-Cas9 involved targeting the Abnormal Cytokinin Response1 Repressor1 (*ARE1*) gene in the wheat variety 'ZhengMai 7698.' Since ARE1 suppresses Ferredoxin-dependent Glutamate 2-Oxoglutarate Aminotransferase (Fd-GOGAT), an enzyme crucial for nitrogen assimilation, its mutation led to improved nitrogen starvation tolerance, delayed senescence, and higher yield, highlighting the potential of CRISPR-Cas9 in enhancing nitrogen use efficiency in wheat (Zhang et al., 2021).

Genome editing has also been employed to improve wheat grain composition. The CRISPR-Cas system has been used to target α-gliadin genes, reducing gluten content in wheat grains (Sánchez-León et al., 2018). Additionally, mutation of the *TaSBEIIα* gene through CRISPR-Cas9 has successfully generated high-amylase wheat with significantly improved starch content (Li et al., 2021). The functions of four grain-quality-related genes - *pinb, waxy, ppo, and psy* - associated with wheat grain hardness, starch quality, and dough color were examined. Using *Agrobacterium*-mediated CRISPR delivery, new allelic variations of these genes were introduced into modern wheat lines (Zhang et al., 2021). Moreover, CRISPR-Cas9 has been applied to modify the Inositol pentakisphosphate 2-kinase 1 (*TaIPK1*) gene, reducing phytic acid levels in wheat grains to enhance iron and zinc biofortification (Ibrahim et al., 2022).

3.20 Site directed mutagenesis by using transgenic pollen

Site-directed mutagenesis is a powerful tool for validating gene function experimentally and accelerating plant breeding by generating new genetic variation. However, its application in wheat is challenging due to the crop's high genomic redundancy and genotype-specific DNA transfer methods (Kelliher et al., 2019a). The era of transgenesis enabled the development of desired traits by introducing recombinant DNA into elite backgrounds, but its adoption has been hindered by lengthy and costly regulatory evaluations stemming from exaggerated perceptions of method-specific risks. Alternatively, meiotically recombinant and genetically fixed doubled haploids have

proven effective for advancing crop improvement (Kalinowska et al., 2019). *In planta* haploid induction via uniparental genome elimination has been demonstrated in various species: in *Arabidopsis* through modifications of *CENTROMERIC HISTONE 3 (CENH3)* (Ravi & Chan, 2010), in maize and rice via knockout of a sperm-specific phospholipase gene (Kelliher et al., 2017), and in wheat through intergeneric crosses with maize (Laurie & Bennett, 1988). Moreover, the combination of haploid induction with site-directed mutagenesis has been reported in *Arabidopsis*, maize, and wheat (Kelliher et al., 2019b).

The non-GM-based transfer of alien genes in cereals requires, as a first step, the efficient generation of inter-generic or interspecific hybrids (Friebe et al., 1996). The first viable hybrid plants between bread wheat (*Triticum aestivum*) as female and barley (*Hordeum vulgare*) as male were produced by the end of the 1970s (Fedak, 1980; Islam et al., 1981). Uniparental chromosome elimination is a common process in interspecific and intergeneric cereal hybrids. When wheat (♀) is crossed with barley (♂) parent, the resulting F1 hybrids plants are either maternal haploid or partial hybrids containing at least one barley chromosome (Polgári et al., 2019). Thus wheat (♀) x barley (♂) cross gives an opportunity to achieve transgene free genome editing in wheat. The process may involve pollinating a wheat plant with pollen from a barley plant containing a transgene suitable for wheat gene editing. The genome of the resulting haploid progeny is modified by the function of the transgene transferred by the crossing, while the gene-editing transgene is eliminated along with the chromosomes of the barley.

Despite considerable advances in wheat-barley hybridization and genome editing technologies, several critical limitations persist in current research. First, while interspecific crosses between wheat and barley offer potential for trait introgression, the lack of efficient, high-throughput methods for characterizing hybrid genomes remains a major bottleneck. Traditional cytogenetic techniques like GISH and FISH, though reliable, are labor-intensive and impractical for large-scale screening, creating a need for rapid molecular tools. Second, although CRISPR/Cas9 has revolutionized plant genome editing, its application in polyploid species like wheat faces challenges due to low transformation efficiency and most of the elite verities are recalcitrant to *Agrobacterium* mediated transformation. Transgene integration puts another hurdle to release a variety as in most countries it is not permissible. Current transgene-free editing

methods often yield inconsistent results or require specialized delivery systems that may not be universally applicable.

This knowledge gap hinders the reliable application of wide hybridization in breeding programs. Although haploid induction has already been combined with genome editing between wheat and maize, this approach has not been sufficiently successful, as the Hi-Edit system suffers from very low editing efficiency and faces challenges in genome duplication of wheat (Kelliher et al., 2019).

To address these limitations, this study proposes an integrated strategy combining molecular karyotyping with innovative genome editing approaches. The development of a multiplex PCR-based system for chromosome identification would provide a scalable alternative to cytogenetic methods, enabling efficient screening of hybrid populations. Simultaneously, the use of transgenic barley pollen to deliver CRISPR/Cas9 components into wheat represents a novel, DNA-free editing strategy that could overcome current barriers.

The specific research questions guiding this investigation include: (1) Can a robust MPCR system be developed for rapid and accurate chromosome identification in wheat-barley hybrids and their wild relatives? (2) Can CRISPR/Cas9 be effectively delivered via barley pollen to induce targeted mutations in wheat, followed by backcrossing to recover barley chromosome less and transgene-free edited plants?

4. OBJECTIVES

Based on the literature and context reviewed in Chapter 3, the following objectives were formulated for this thesis.

The objectives of this study were:

- Establish a DNA-free genome editing platform for wheat by combining wheat × barley hybridization with CRISPR/Cas9 technology.
- Generate F1 wheat × barley hybrid plants containing a CRISPR/Cas9 cassette to induce targeted mutations in the *mlo* allele of wheat.
- Perform backcrossing of F1 hybrids with wheat to eliminate barley chromosomes and obtain transgene-free, edited wheat plants.
- Develop a bioinformatic pipeline to analyze wheat and barley reference genomes and design chromosome-specific PCR primers for precise detection of individual chromosomes in wheat, barley, and their hybrids. This pipeline can be extended to other crops undergoing interspecific or intergeneric hybridization.
- Establish and optimize a routine, fast, and cost-effective Multiplex PCR (MPCR) assay using these primers to assess chromosome composition in wheat, barley, their hybrid progeny, and related species within *Triticum* and *Hordeum* genera for trait improvement.

5. MATERIALS AND METHODS

5.1 Plant materials

For the MPCR analysis and transgene free genome editing a doubled haploid (DH) line of spring wheat 'M1' (*Triticum aestivum*, 2n = 6x = 42, AABBDD), originating from Sichuan, served as the female parent, was crossed with male parent two-row spring barley 'Golden Promise' (*Hordeum vulgare*, 2n = 2x = 14, HH). The parent plants were cultivated in a growth chamber (Conviron, Winnipeg, Canada) under controlled conditions, including a 16 h- photoperiod with a light intensity of 150–500 µmol•m^{-2} s^{-1} PPFD and a constant temperature of 18 °C. Flowering periods were synchronized by staggered planting. All other wheat and barley genotypes used in the MPCR analyses were grown in Jiffy peat pellet (Jiffy – 7, 33mm, Jiffy International AS, Kristiansand, Norway).

Barley (*Hordeum vulgare*) 'Golden Promise' plants were grown in growth cabinet (Versatile Environmental Test Chamber MLR-350; Sanyo, Tokyo, Japan) under 15°C daytime and 12°C night temperatures with 16-h light (50 µE $m^{-2}s^{-1}$) and 8-h dark periods to obtain explants for *Agrobacterium*-mediated genetic transformation.

5.2 Generation of CRISPR/Cas9 construct and barley transformation

We opted for the pHUE411 vector system described by Xing et al. (2014). To facilitate the detection of the integrated T-DNA, a *35S::DsRed* construct was inserted into the *Pme*I site of the pHUE411 vector, which was amplified from the pC61KdsRED vector (Kis et al., 2019) generating the pHUER plasmid. The CRISPR/Cas9 vector containing a single sgRNA was prepared as described in (Xing et al., 2014). The presence and accuracy of the introduced sgRNA sequence in the generated vector were confirmed by sequencing. The selected target sequence included a restriction cleavage site, *CaC*8I, overlapping with the CRISPR/Cas9 target sites to facilitate mutation detection. For proof of concept, we chose *MLO* gene. Based on article (Y. Wang et al.,

2014b), the sequence of the *MLO* gene was identified. Using Ensembl Plants (https://plants.ensembl.org/index.html), we blasted the sequence and extracted the sequences of all three homeoalleles with the following gene IDs: 5A-TraesCS5A02G494700, 4D-TraesCS4D02G319000, and 4B-TraesCS4B02G322600. While Y. Wang et al., 2014 guide only targeted the *TaMLO-A* gene and has mismatches with *TaMLO-B and TaMLO-D* genes we designed a guide capable of targeting *TaMLO-A, TaMLO-B, and TaMLO-D* genes. The alignments are presented in the appendices (Suppl. Figure S1).

Immature barley embryos were transformed by *A. tumefaciens* (AGL1 strain) as described in (Kis et al., 2016), harboring the pHUER vectors containing the *Cas9* gene under the control of the maize ubiquitin (*Ubi*) promoter and one sgRNA specific for all three *MLO* homeoalleles. Transgenic plants that originated from the same callus were considered as sibling lines. The presence of the transgene was detected by DsRed marker protein fluorescence followed by PCR reaction using primer pairs specific for the *Ubi* promoter (Suppl. Table S6). Direct DNA was extracted in a similar way mentioned in (materials and methods section 5.4).

5.3 Crossing and treatments

The wheat spikelets were trimmed at the tip 2-3 days before anthesis. The wheats maternal ears were emasculated at least 2-3 days before anthesis. After removing the internal (up from 3rd). florets from each spikelets, the upper 1/3 of the bracts of the remaining two major flowers are cut back with scissors. Green unripen anthers were carefully removed manually by needle tipped tweezers. To prevent unintended fertilization, the emasculated ears were covered with isolating cellophane bags till visible receptivity of the stigmas. On the day of flowering, the mature barley spikes were collected and after opening the florets by shortening the bracts by scissors put them into warm water containing jar, to induce the anthesis. Pollination was performed manually by brushing barely spikes onto the wheat flowers. The cellophane bags were temporarily removed during pollination and then resealed. Following pollination, 1.5 mL of seed development inducing solution (SDIS) containing final concentration of 1% DMSO, 0.01% (4.5 µM) 2,4-dichlorophenoxyacetic acid, and (500 µM) 5-azacitidine (a chemically modified nucleoside analogue that inhibits the transfer of methylation patterns to newly synthesized DNA strands hence

reactivate epigenetically silenced genes in the developing embryo and endosperm) was injected into the first internode beneath the pollinated spike to support the caryopsis development.

Fourteen days post-fertilization, developing caryopses were removed from the pollinated spikes. After surface sterilization (see section 5.3), the developing hybrid embryos were carefully picked after a longitudinal excision of pericarp.

5.4 Embryo rescue

Fourteen days after fertilization, caryopses were collected from the pollinated spikes. These seeds were sterilized by immersing them in 70% ethanol for 3 minutes, followed by 2% NaOCl (hypochlorite) for 20 minutes. After sterilization, the seeds were rinsed three times with sterile Milli-Q water. The disinfected seeds were then slit longitudinally using a dissecting needle, and their contents were examined. The embryos identified during this process were germinated and regenerated on N6D medium (Chu et al., 1975). If the number of embryos was insufficient, they were first cultured on callus induction medium, followed by transition medium, and finally placed on regeneration medium (Harwood et al., 2009).

5.5 DNA extraction

Total DNA was extracted from young leaf and root samples using a direct DNA extraction method. Leaf pieces (~5 × 5 mm) or root segments (~2 cm) were placed into a 1.5 mL Eppendorf tube containing 100 µL of Extraction Solution (E7526, 24 mL, Sigma-Aldrich, St. Louis, MO, USA) and a 3 mm stainless-steel bead (Qiagen Sciences, Germantown, MD, USA). The samples were homogenized in a mixer mill (Bullet Blender Storm Pro, Next Advance, Troy, NY, USA) at speed setting 8 for 1 minute. The mixture was incubated at 95 °C for 12 minutes in a dry heat block, then cooled on ice for 1 minute. Afterward, 100 µL of Dilution Solution (D5688, 12 mL, Sigma-Aldrich) was added. The samples were vortexed and centrifuged at 18,000 × g for 1 minute. The supernatant (100 µL) was transferred to a new 1.5 mL Eppendorf tube, and the DNA was stored at -20 °C until use.

5.6 Generation of Multiplex PCR primers for the wheat and barley genomes

I would like to thank Dr. Levente Kontra for their contribution to the design and in silico testing of the PCR primers used in this study. Their expertise and support were essential to the successful completion of this part of the research.

To begin the process, the reference genomes of wheat (*Triticum aestivum* 'Chinese Spring', IWGSC RefSeq v1.0) and barley (*Hordeum vulgare* 'Golden Promise', GPv1) were downloaded from the Ensembl database. These reference genomes were fragmented into all possible 20-bp long sequences, known as 20-mers, using the Jellyfish tool (Marçais & Kingsford, 2011). The generated 20-mers were then compared against the 20-mers obtained from three additional genome assemblies, specifically those of the wheat cultivars 'Weebill 1' and 'Claire', as well as the reference genome (V3) of the 'Morex' barley variety. To ensure uniqueness, any 20-mers discarded that appeared more than once in any of the genomes or that showed exact matches in both orientations across all genomes were discarded. Following this, we filtered out 20-mers containing fewer than three different types of nucleotides, as these sequences are generally less useful for specificity in primer design. We further refined the selection by focusing on 20-mers with a GC content of around 60%, a parameter chosen to enhance PCR efficiency. Since PCR primers can sometimes anneal incompletely, we randomly selected 100 pairs per chromosome for mapping to the reference genomes. This mapping was performed using PatMaN software (Prüfer et al., 2008), allowing up to two mismatches to ensure flexibility in primer alignment. Primer pairs that matched the target genome more than once within a 1000-bp region, in either orientation, were removed to avoid amplification of unintended regions. The remaining primer pairs were further refined based on their amplification product lengths. For each chromosome, we grouped the amplified fragments into specific size ranges with a ±5 bp tolerance: chromosome 1 (100 bp), chromosome 2 (150 bp), chromosome 3 (200 bp), chromosome 4 (250 bp), chromosome 5 (300 bp), chromosome 6 (350 bp), and chromosome 7 (400 bp). These carefully selected primer pairs were then divided into four pools: plex-A, plex-B, and plex-D for the wheat A, B, and D sub-genomes, respectively, and plex-H for the barley genome. To minimize off-target effects, we conducted an *in silico* analysis of all

primer pairs in each plex group. We iteratively removed primer pairs that resulted in the highest number of unspecific products, repeating this process until no significant off-targets remained. For further validation, a random set of primer pairs for each chromosome was manually verified using Ensembl BLAST. In cases where the specificity needed improvement, we made slight adjustments, such as shifting the primer positions by a few nucleotides or extending their lengths. After individual PCR tests on the reference genomes, we evaluated the broader applicability of each plex *in silico* by including an additional 16 bread wheat assemblies (IWGSC_refseqv1.0, CAJRHR01, CAJEVV01, CAJEWR01, CADDYP01, CAJEWS01, CAJEWQ01, CADDYN01, CADDYO01, CADDYM01, CADDYY01 CAJEVU01, CAJEVW01, CAJFAH01, CAJEWO01, CAJFCQ01), and two barley genome assemblies (CAJHDD01, PRJEB34496) along with genomes of wild and progenitor species, to confirm the versatility and robustness of our designed primers.

5.7 PCR and Multiplex PCR

Single PCR reactions were conducted in 20 µL reaction volumes, consisting of 1 µL of direct total DNA extract as the template, 4 µL of 5X Phusion Green HF Buffer (F-538, Thermo Scientific, Waltham, MA, USA), 0.5 µM of each forward and reverse primer, 4 µM of dNTPs (Thermo Scientific), 0.4 U of Phusion Hot Start II High-Fidelity DNA Polymerase (F-549, Thermo Scientific), and nuclease-free water to bring the total volume to 20 µL. The PCR cycling program was optimized as follows: an initial denaturation step at 98 °C for 3 minutes, followed by 32 cycles of denaturation at 98 °C for 10 seconds, annealing at 65 °C for 15 seconds, and extension at 72 °C for 10 seconds. After the cycling, the final extension step was performed at 72 °C for 5 minutes, and the reactions were held at 4 °C. The cycling condition for *mlo* gene PCR was as follows: an initial denaturation step at 98 °C for 3 minutes, followed by 30 cycles of denaturation at 98 °C for 10 seconds, annealing at 67 °C for 15 seconds, and extension at 72 °C for 10 seconds. After the cycling, the final extension step was performed at 72 °C for 5 minutes, and the reactions were held at 4 °C.

For the Multiplex PCR (MPCR) assay, which targets all seven chromosomes of each wheat sub-genome (A, B, and D) as well as the barley H genome, the reaction setup was slightly modified. The MPCR reactions were performed in 20 µL volumes using the 2X Phusion U Green

Multiplex PCR Master Mix (F-564, Thermo Scientific). Each reaction contained 10 µL of the master mix, 0.3 µM of each primer, 1 µL of total DNA extract, and nuclease-free water to adjust the final volume. In certain experiments, variations in the reaction setup were introduced. For example, we utilized the 5X Phusion Green HF Buffer in combination with Phusion Hot Start II High-Fidelity DNA Polymerase, or alternatively, Phire Hot Start II DNA Polymerase (F-122, Thermo Scientific). In such cases, either the 5X Phire Green Reaction Buffer (F-527, Thermo Scientific) or the 5X Phusion Green HF Buffer were used, as in the single PCR reactions, but with the primer concentrations adjusted to 0.3 µM for each primer.

The thermal cycling conditions for the MPCR reactions using the Phusion U Green Multiplex PCR Master Mix were as follows: an initial denaturation at 98 °C for 3 minutes, followed by 32 cycles of denaturation at 98 °C for 10 seconds, annealing at 65 °C for 30 seconds, and extension at 72 °C for 10 seconds. A final extension was performed at 72 °C for 5 minutes, followed by a hold at 4 °C. These cycling conditions were applied for plex-A, plex-B, and plex-D, corresponding to the wheat A, B, and D sub-genomes. However, for plex-H (targeting the barley genome), the annealing temperature was adjusted to 68 °C. In all other cases, when alternative enzyme and buffer combinations were used, an annealing temperature of 68 °C was consistently applied across all primer plexes. The PCR reactions were run on a Mastercycler® nexus gradient thermal cycler (Eppendorf, Hamburg, Germany) to ensure precise temperature control and reproducibility.

5.8 Gel electrophoresis

After the individual and MPCR reactions, the resulting individual PCR products and MPCR products were separated by electrophoresis on 1.2% and 2% (w/v) agarose gels, respectively, containing ethidium bromide. The gels were run in 1X TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) at 120 V for 30 minutes for individual PCR and at 150 V for 30 minutes for MPCR to achieve clear separation of the DNA fragments. A GeneRuler™ 100 bp Plus DNA Ladder (Thermo Scientific) was used as a molecular size marker to determine the size of the PCR products. After electrophoresis, the gel images were captured using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA), ensuring high-quality visualization of the amplified

DNA fragments. These steps allowed us to confirm the successful amplification of the target regions and assess the specificity and efficiency of the designed primers across different genome assemblies.

5.9 Genomic (GISH) and fluorescence (FISH) *In situ* hybridization

I gratefully acknowledge Dr. Sepsi Adel for conducting the cytogenetic work, including chromosome preparation and the GISH/FISH experiments, which were essential for the justification of the analysis of wheat × barley hybrids in this study.

To prepare for mitotic chromosome analysis, the roots of the wheat × barley F1 hybrids were harvested directly from their growth pots. The collected roots underwent a pre-treatment in ice-cold water, which contained melting ice, for a minimum of 24 hours. This cold treatment served to arrest cell division at the metaphase stage, making the chromosomes more visible for later examinations. Following this pre-treatment, the roots were fixed using Clarke's fixative, a solution composed of a 3:1 (v/v) mixture of absolute ethanol and glacial acetic acid. The roots remained in this fixative for five days at 37 °C to ensure thorough penetration and preservation of the cellular structures. After fixation, the roots were stained with 1% (w/v) acetocarmine (C1022, Sigma-Aldrich), a classic stain used for visualizing chromosomes. To preserve the stained roots, they were stored in Clarke's fluid at -20 °C for two weeks, after which they were ready for chromosome preparations. To generate the *Genomic In Situ Hybridization* (GISH) probe, total DNA from barley (variety 'Morex') was extracted from fresh young leaves using the standard CTAB (cetyltrimethylammonium bromide) extraction method. The extracted barley DNA was then fragmented into smaller pieces of approximately 300–500 base pairs (bp) by heating it in a pressure cooker for 6 minutes. One microgram of this fragmented barley DNA was labelled using the AF594 NT Labelling Kit (PP-305 L-AF594, Jena Bioscience, Jena, Germany) through a process known as nick-translation, which incorporates fluorescent markers into the DNA. This labelled barley DNA was subsequently used as the GISH probe, with 40–50 ng of labelled DNA applied per microscope slide. In parallel, a Fluorescent *In Situ* Hybridization (FISH) probe was prepared by PCR amplification of the barley 5S rDNA coding region along with its flanking non-coding sequences. The amplified product was then labelled directly using the AF488 NT Labelling Kit

(PP-305 L-AF488, Jena Bioscience) through the same nick-translation process. This FISH probe allowed for specific identification of the 5S rDNA regions in the chromosomes. The detection and identification of barley chromosomes in the wheat-barley hybrids were conducted simultaneously using both the GISH and FISH techniques. Prior to hybridization, the chromosome preparations were treated with an enzymatic digestion using a 50 mg/mL solution of pepsin dissolved in 1 mM HCl for 1–2 minutes, which helped remove excess proteins that could obscure the chromosomes. This was followed by a post-fixation step using 4% (w/v) paraformaldehyde (PFA), prepared by diluting a 16% stock solution (28,908, Thermo Scientific), for 10 minutes to ensure that the chromosome structures were adequately preserved. For the hybridization process, a hybridization mixture was prepared, consisting of 60% (v/v) deionized formamide (F9037, Sigma-Aldrich), 10% (w/v) dextran sulphate (D8906, Sigma-Aldrich), and 2X Saline Sodium Citrate (SSC) buffer. This mixture was crucial for facilitating the specific binding of the labelled DNA probes to their complementary sequences on the chromosomes. A total of 17 µL of the hybridization mixture was applied to each slide, which contained 40–50 ng of both the GISH and FISH probes. To block non-specific hybridization signals, an excess of unlabelled wheat DNA (in a 30:1 ratio to the GISH probe) was added to the mixture. The probe mixture underwent an initial denaturation step at 85 °C for 8.5 minutes to separate the DNA strands, followed by an additional denaturation step at 75 °C for 3 minutes after being applied to the slides. This ensured that the probes would bind specifically to their target sequences during hybridization. Following hybridization, post-hybridization washes were performed to remove unbound probes, and the slides were then mounted with 24 × 32 mm coverslips. A total of 12 µL of Vectashield antifade solution containing DAPI (H1200, Vector Laboratories, Burlingame, CA, USA) was added to the slides to protect the fluorescent signals from fading and to counterstain the DNA. Finally, the chromosome images were captured using an SP8 confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany), which was equipped with an HC PL APO CS2 63×/1.40 oil immersion objective. This high-resolution imaging system allowed for precise visualization and documentation of the chromosomes, providing clear images of the hybridization patterns for further analysis.

5.10 PCR amplicon purification and sequencing

PCR products amplified from the respective DNA templates using primers specific to the target regions were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany), strictly following the manufacturer's protocol. The concentration and purity of the purified PCR products were determined using a Nanodrop spectrophotometer (ThermoFisher Scientific, USA). All the amplicons were directly purified and sent for sequencing but for amplicons from the 1A, 1B, 1D, and 1H chromosomes were first cloned into the pJET1.2/blunt cloning vector system (ThermoFisher Scientific, USA) by following the manufacturer's instructions. Because they were rather small to get better sequencing results. The ligation mixture was transformed into competent *Escherichia coli* DH5α cells using the heat shock method (42 °C for 45 seconds), and plasmids were then extracted from the transformed colonies. Sequencing purified PCR amplicons and plasmids in case of 1A, 1B, 1D, and 1H chromosome was performed using the respective forward and reverse primers. The purified plasmid and PCR samples were sent for sequencing to Eurofins Genomics (Germany).

5.11 Detection of targeted mutations

Following PCR, restriction enzyme digestion was performed to analyze potential mutations. A 10 µL digestion reaction was prepared, consisting of 3 µL of unpurified PCR product, 1 µL of 10× rCutSmart™ Buffer , 0.5–1 µL of *Cac*8I enzyme (as recommended by the supplier), and 5.5–6 µL of nuclease-free water. The reaction was incubated at the optimal temperature for the enzyme, typically 37°C, for 3 hours or overnight, followed by enzyme inactivation if required (e.g., 65°C for 20 minutes). The digested products were then analyzed by gel electrophoresis using a 1.2% agarose gel stained with ethidium bromide. The digestion patterns were used to determine the presence of mutations.

6. RESULTS

6.1 Computational analysis and development of Multiplex PCR primers targeting specific chromosomes

To develop Multiplex PCR (MPCR) primer sets capable of specifically detecting individual chromosomes in wheat and barley, we began by breaking down two reference genomes into approximately 18.68 billion 20-base-pair (bp) sequences. These sequences were systematically screened for key parameters, including uniqueness (assessed through exact matches), sequence complexity, and GC content, to identify the most suitable primer candidates. This filtering process reduced the initial pool to 35.28 million high-quality sequences. To enable precise chromosome-specific detection, we further selected sequences based on specific inter-primer distances. This refinement resulted in 270,413 primer pairs, which were then organized into four MPCR subsets: plex-A (targeting the wheat A sub-genome), plex-B (wheat B sub-genome), plex-D (wheat D sub-genome), and plex-H (barley genome). Despite successfully designing these primer sets, initial PCR testing revealed non-specific fragment amplification in several cases.

To address these shortcomings, we enhanced our protocol by incorporating additional genome data and refining filtering parameters. Specifically, we included three more genome assemblies (two wheat and one barley) to exclude sequences with non-unique or multiple occurrences. We also allowed mismatches and applied stricter controls for off-target and cross-off-target amplification (Figure 6). Through this improved screening process, the initial pool of 18.68 billion sequences was reduced to 287.29 million potential candidates with a maximum of two mismatches. Further filtering eliminated primer pairs prone to off-target amplification, and sequences were grouped by defined size ranges. From this refined set, we randomly selected 100 primer pairs per chromosome from the remaining 9,437 pairs.

Using *in silico* modeling, we simulated the use of all 700 primer pairs (per plex) in PCR experiments, allowing for two mismatches. Iterative refinement was performed by systematically removing primer pairs with the highest off-target amplification rates until a final cross-off-target-

free set was achieved (Suppl. Table S2). Certain chromosomes posed challenges due to limited primer pair availability, for instance, chromosome 2D had only three suitable pairs, whereas others offered a more abundant selection.

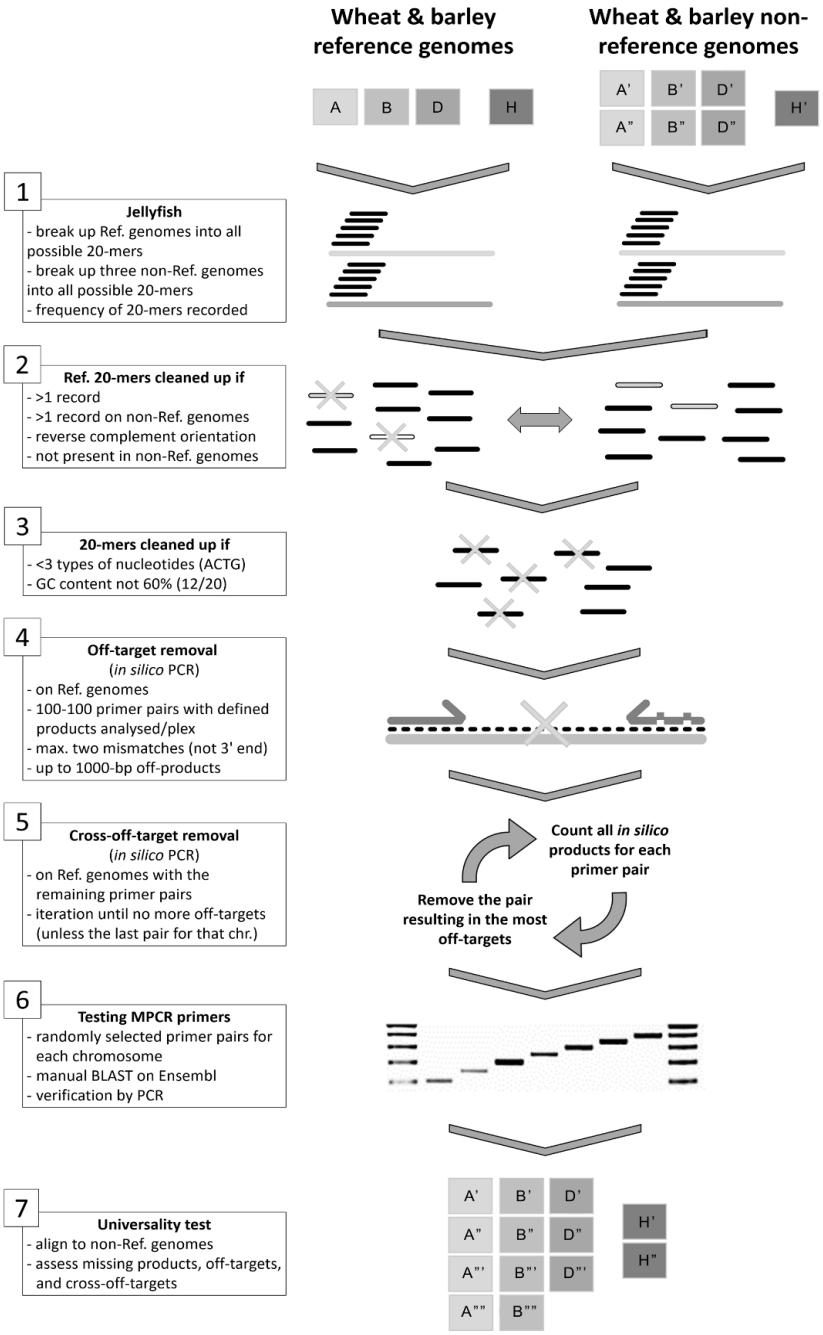

Figure 6: Illustrative representation of the refined chromosome-specific MPCR primer design protocol. A, B, D and H are reference genomes. A′, A″… B′, B″… D′, D″…and H′, H″ are non-reference genomes. (Ali et al., 2024).

The finalized primer sets (Suppl. Table S3, Fig. 7) underwent thorough validation through manual PCR testing and *in silico* cross-referencing against sequenced genomes from 16 bread wheat and two barley cultivars. The positions of the primers were also determined and illustrated on reference genomes of wheat and barley using BLAST (Figure 7). A few anomalies were observed during validation. For example, primers 350bp_F_6A and 350bp_R_6A produced two distinct 346-bp amplicons from separate locations in the 'Robigus' wheat genome. This discrepancy may be attributed to incomplete genome assembly in this cultivar, potentially representing a bioinformatic artifact.

In another case, primers 150bp_F_2B and 150bp_R_2B did not predict an amplicon in the genome of 'LRPB Lancer' wheat through bioinformatic analysis. However, PCR verification confirmed the expected product using DNA from this cultivar.

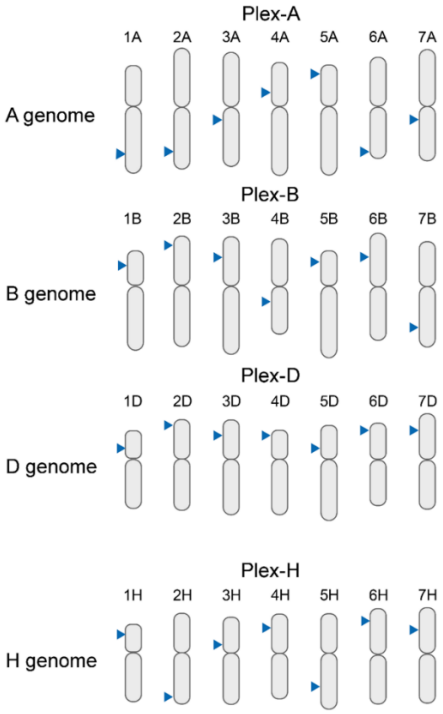

Figure 7: Diagrammatic representation of target chromosomes and Multiplex PCR primer set positions. The upper three panels illustrate the distribution of specific MPCR primers across the seven chromosomes of wheat A, B, and D sub-genomes (designated as plex-A, plex-B, and plex-D, respectively). The lower panel displays the arrangement of specific MPCR primers on the seven barley chromosomes (plex-H). Arrowheads indicate the precise chromosome locations of the primers (Ali et al., 2024).

Additionally, the primer pair 250bp_F_4D and 250bp_R_4D produced a 242-bp by-product from chromosome 4B from wheat cultivars 'LRPB Lancer', 'Paragon', 'SY Mattis', and 'Julius'. Sequence analysis revealed a mismatch at the 3' end of the 250bp_F_4D primer, which likely disrupted amplification, as corroborated by sequencing data (Figure 8).

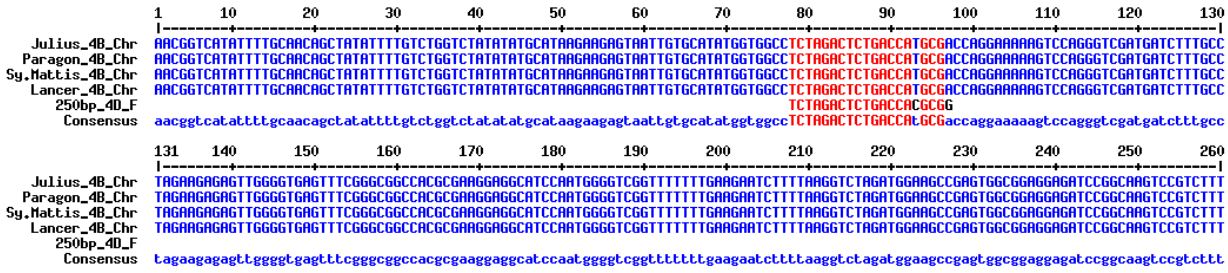

Figure 8: Sequence alignment analysis of chromosome 4B in *Triticum aestivum* Julius, *Triticum aestivum* Paragon, *Triticum aestivum* Sy Mattis, and *Triticum aestivum* Lancer using the 250bp_4D_F primer. The analysis revealed a mismatch at the 3' end of the 250bp_4D_F primer, likely disrupting amplification.

6.2 Assessment of the designed MPCR primer sets

The amplicons generated by the designed MPCR (Multiplex PCR) primer sets were specifically tailored to produce distinct product sizes, increasing stepwise for each chromosome across the (sub-)genomes. These incremental sizes were strategically assigned as follows: chr1 – 100 bp, chr2 – 150 bp, chr3 – 200 bp, chr4 – 250 bp, chr5 – 300 bp, chr6 – 350 bp, and chr7 – 400 bp. This systematic size progression ensured clear differentiation of PCR products during electrophoretic separation, simplifying downstream analysis and interpretation.

To comprehensively verify the specificity and sensitivity of the designed primer pairs, we first conducted individual PCR tests using DNA templates derived from reference genomes. The results showed precise amplification of products corresponding to the expected sizes in all tested scenarios (Figure 9A). Each primer set consistently generated a single, well-defined amplicon without any nonspecific by-products, indicating their high target affinity and amplification efficiency.

In particular, the wheat-specific primers, derived from the cultivar ‘Chinese Spring,’ successfully detected their corresponding wheat chromosomes with remarkable precision. Simultaneously, the barley-specific primers demonstrated no cross-reactivity with wheat genomic DNA, further emphasizing their species-specificity (Figure 9A). To validate these results at the molecular level, we performed sequence analyses on all amplified products. These analyses confirmed that each of the 28 PCR products matched the sequences predicted through *in silico* bioinformatic analysis (Suppl. Table S5). This correlation between experimental and computational data provides robust evidence that the primer sets are highly reliable, with no off-target amplification events.se

Following these initial verifications, we evaluated the performance of the chromosome-specific primer sets in a more complex MPCR setup using total genomic DNA extracted from two widely studied reference cultivars: ‘Chinese Spring’ (wheat) and ‘Golden Promise’ (barley). In the Multiplex reaction setup, primer sets were grouped according to their corresponding (sub-) genomes, and the results were analyzed based on their chromosomal origin. The MPCR experiments (Figure 9B) yielded clear, distinct bands for all target chromosomes from both wheat and barley genomes. Importantly, the size of the amplified products was consistent with those observed in the single PCR tests (compare Figure 9A and Figure 9B), reaffirming the reproducibility and accuracy of the MPCR assay.

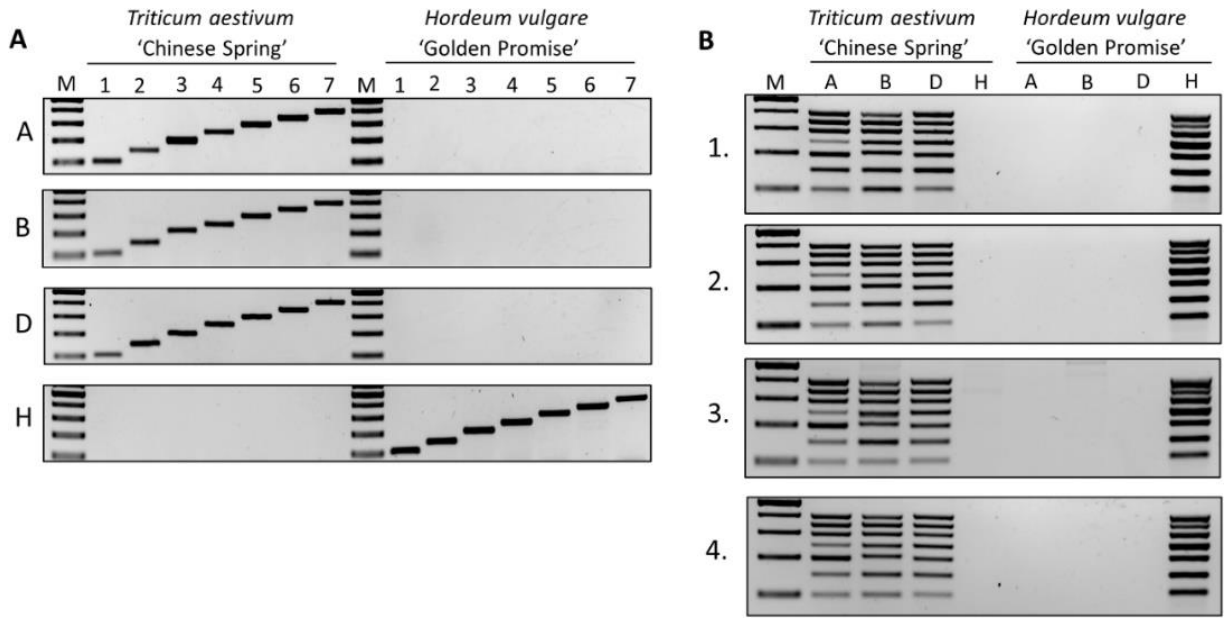

Figure 9: A: Single PCR amplifications were performed using primers specific to chromosomes 1–7 from the A, B, and D sub-genomes of wheat ('Chinese Spring') and the H genome of barley ('Golden Promise'). Lanes 1–7 represent the PCR amplicons corresponding to each chromosome, while lane M shows the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder). The reactions were carried out using Phusion Green HF buffer and Phusion Hot Start II High-Fidelity DNA Polymerase to ensure high specificity and accuracy of amplification. **B:** MPCR amplification was conducted for chromosomes 1–7 of the A, B, and D sub-genomes of wheat ('Chinese Spring') and the H genome of barley ('Golden Promise') using various buffer and DNA polymerase combinations to evaluate protocol flexibility. The following conditions were tested: 1 Phusion U Green Multiplex PCR Master Mix. 2 Phusion Green HF Buffer with Phusion Hot Start II High-Fidelity DNA Polymerase. 3 Phire Green HF Buffer with Phire Hot Start II High-Fidelity DNA Polymerase. 4 Phusion Green HF Buffer with Phire Hot Start II High-Fidelity DNA Polymerase. Lane M represents the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder). These results confirm the robustness and adaptability of the MPCR protocol across different buffer-polymerase systems while maintaining specificity and accurate product sizes (Ali et al., 2024).

A critical observation from the MPCR experiments was the absence of nonspecific products and lack of cross-reactivity between wheat and barley DNA. This result underscores the precision of the primer sets and their suitability. To assess the technical flexibility and robustness of the MPCR protocol, we further tested the method using four commercially available buffer-DNA polymerase systems. Despite differences in the composition and properties of these systems, all four supported successful DNA amplification without introducing nonspecific products (Figure 9B). This indicates that the MPCR protocol is adaptable to a range of commonly used commercial

reagents, making it highly versatile for laboratories with varying resources and preferences. By enabling simultaneous detection of multiple targets in a single reaction, the MPCR approach reduces reagent consumption, minimizes labor-intensive steps, and shortens experimental timelines. These attributes make it particularly advantageous for high-throughput applications in both research and diagnostic settings.

6.3 Broad applicability of MPCR across a wheat and barley panel

Total DNA samples were extracted from 14 wheat cultivars and five barley cultivars (Suppl. Table S1: below panel) and analyzed through separate MPCR reactions targeting the wheat A, B, and D sub-genomes as well as the barley H genome (Figure 10: panels A to H, respectively). These MPCR reactions yielded distinct and well-defined band patterns corresponding to the expected sizes for all (sub-)genomes. The specificity of the primer sets was confirmed by the clear and consistent amplification of the target regions, with no non-specific cross-reactions observed across the different wheat and barley cultivars.

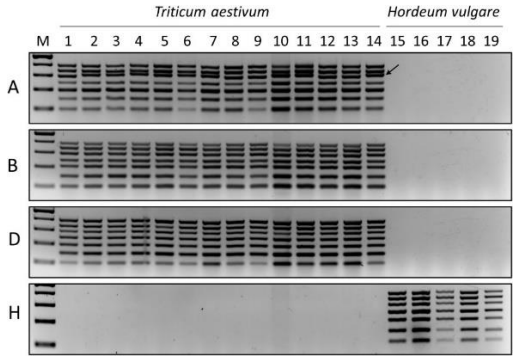

Figure 10: MPCR amplification was conducted on chromosomes 1–7 of the A, B, and D sub-genomes from 14 wheat cultivars (1: Chinese Spring reference genome, 2: Bobwhite, 3: Fielder, 4: Bánkúti 1201, 5: LRPB Lancer, 6: CDC Stanley, 7: Paragon, 8: SY Mattis, 9: Julius, 10: Cadenza, 11: Weebill 1, 12: Claire, 13: Robigus, 14: Jagger) and the H genome of five barley cultivars (15: Golden Promise reference genome, 16: Morex, 17: Igri, 18: California Mariout, 19: Esperanza). The amplification resulted in distinct, well-defined bands for all (sub-)genomes, with arrows indicating an increased size of the 5A-specific product in several wheat cultivars. The molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder) is denoted by M, and the reactions were carried out using Phusion Green HF Buffer and Phusion Hot Start II High-Fidelity DNA Polymerase for optimal amplification performance and specificity (Ali et al., 2024).

One notable observation was the minor variation in the size of the 5A chromosome-specific amplicon in several wheat cultivars, including 'Bobwhite,' 'Fielder,' 'Bánkúti 1201,' 'LRPB Lancer,' 'CDC Stanley,' 'Paragon,' 'Cadenza,' 'Weebill 1,' and 'Jagger' (Figure 10: panel A, arrow). While the amplification was still specific, a slight increase in product size was noted in these cultivars, which suggested a potential genomic variation affecting the amplification pattern. Further *in silico* sequence analysis of these particular amplicons revealed that they contained a 12-bp insertion within the corresponding genomic region (Suppl. Table S4). This insertion was consistent across all the affected cultivars, confirming that the size discrepancy was due to a genomic variation rather than primer inefficiency or non-specific amplification.

Despite this minor variation, the overall results clearly demonstrate that the designed MPCR primer sets are highly specific, yielding clear and reproducible band patterns across a wide range of wheat and barley cultivars. Furthermore, no cross-reactions were observed between the genomes of different cultivars and species, indicating the excellent specificity and reliability of the primer sets for genetic analyses in wheat and barley. These findings highlight the broad applicability and robustness of the MPCR methodology for genetic screening and marker-based analyses in diverse wheat and barley germplasm.

6.4 MPCR primer sets effectively determine the chromosome composition of wheat × barley hybrids.

As a practical application of the designed MPCR primer sets, we evaluated their ability to assess the chromosome composition in 'M1' wheat (♀) × 'Golden Promise' barley (♂) F1 hybrid plants. A total of 16 hybrid plants were regenerated from 18 embryos rescued from 20 pollinated spikes. MPCR analyses of the plants revealed that they all contained the complete set of wheat chromosomes, with one exception: plant No. 14, which exhibited a faint band for the 3B wheat chromosome-specific product (Figure 11). This suggests a slight variation or incomplete amplification for this specific chromosome in this plant, but the overall wheat chromosomal composition was maintained in the majority of plants.

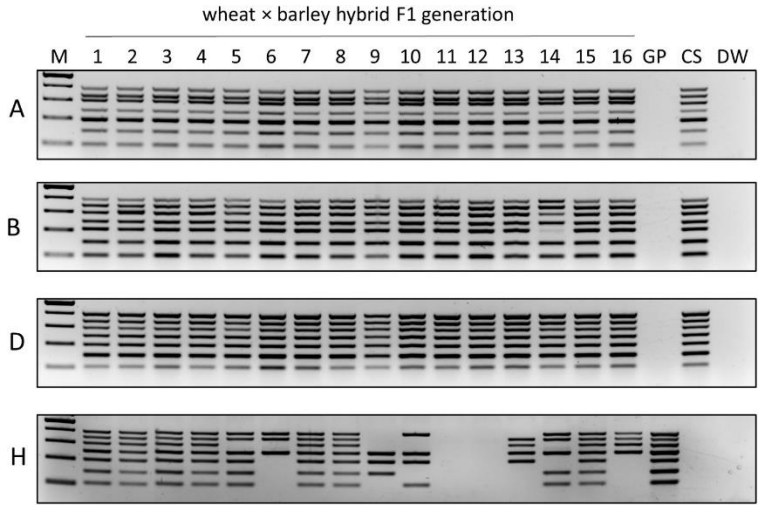

Figure 11: MPCR amplification was performed on chromosomes 1–7 of the A, B, D, and H sub-genomes from wheat × barley hybrids (1–16). M represents the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder), GP refers to the barley cultivar 'Golden Promise,' CS refers to the wheat cultivar 'Chinese Spring,' and DW represents the no-template control. The reactions were carried out using Phusion Green HF Buffer and Phusion Hot Start II High-Fidelity DNA Polymerase to ensure optimal amplification conditions (Ali et al., 2024).

In addition to wheat chromosomes, the barley chromosome composition was also assessed. Seven of the 16 hybrid plants (Nos. 1–4, 7–8, and 15) exhibited the presence of all barley chromosomes in their MPCR profiles, indicating that these plants successfully retained the full barley genome. However, the remaining hybrid plants exhibited varying degrees of chromosome retention: three plants (Nos. 6 and 9) retained five barley chromosomes, while four plants (Nos. 10, 13, and 16) had four barley chromosomes, and one plant (No. 14) retained only three barley chromosomes. The data show that barely chromosomes were maintained and not lost during early development.

Notably, two plants (Nos. 11 and 12) were identified as maternal wheat haploids, as evidenced by the complete absence of barley chromosomes in their MPCR profiles. This result suggests that, while most hybrids contained both wheat and barley chromosomes, these two plants had failed to incorporate any barley genetic material.

Overall, these findings demonstrate that the MPCR primer sets can effectively and efficiently distinguish between the various chromosomes of wheat and barley, even within a hybrid

background. This method provides a cost-effective, highly specific, and practical approach for assessing the chromosomal composition of wheat × barley hybrids, which can be crucial for breeding, genetic studies, and hybrid characterization.

To validate the effectiveness and accuracy of our MPCR technology, we selected two hybrid plants (Figure 11: Nos. 6 and 13) for further analysis using GISH (Genomic *In Situ* Hybridization) and FISH (Fluorescence *In Situ* Hybridization) techniques, employing a barley 5S rDNA-specific probe to identify individual barley chromosomes (Figure 11A and B). Since mitotic chromosomes are typically obtained from root tips, we selected two root tips from each hybrid plant to be processed simultaneously for both *in situ* hybridization and MPCR analysis, allowing us to directly compare the results from both methods.

The GISH-FISH analysis of hybrid plant No. 6 revealed the presence of barley chromosomes 4H + 5H in one root tip, and 4H + 6H + 7H in the other root tip (Fig. 12A). These findings were in full agreement with the results obtained from the MPCR analysis (Fig. 12C), confirming that plant No. 6 is genetically mosaic, with different root tips containing different barley chromosome combinations.

Similarly, for hybrid plant No. 13, GISH-FISH analysis showed that both root tips contained barley chromosomes 3H–6H (Fig. 12A), which was also consistent with the MPCR results (Fig. 12C). These parallel results from GISH-FISH and MPCR analysis indicated that the distribution of barley chromosomes in plant No. 13 was uniform across both root tips.

In summary, the GISH-FISH and MPCR analyses provided identical results for both hybrid plants, confirming that MPCR can accurately and efficiently assess the chromosomal composition of wheat × barley hybrids. This demonstrates that MPCR is a powerful and reliable tool for the rapid screening of hybrid plants, offering a convenient alternative to traditional chromosome identification methods. The consistency between the two techniques highlights the reliability of MPCR for genetic analysis and the characterization of hybrid plants, making it a valuable tool for breeding and genetic research.

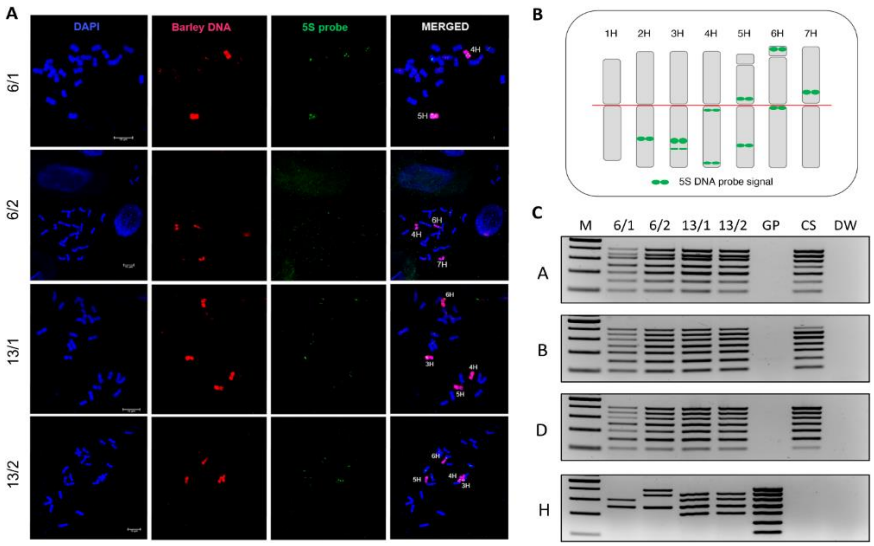

Figure 12: A. Chromosome *in situ* hybridization was performed on two root segments from each of two wheat × barley hybrids (plant Nos. 6 and 13 shown in Fig. 4). The barley genome was detected using GISH (red label), while individual barley chromosomes were identified with FISH using a 5S rDNA-specific probe (green label). The chromosomes were counterstained with DAPI (blue), with scale bars representing 10 µm. **B.** A schematic representation of the position of the 5S rDNA-specific probe on the barley genome is shown, with the red line indicating the location of the centromere. **C.** MPCR amplification of chromosomes 1–7 from the A, B, D, and H sub-genomes of the wheat × barley hybrids (plant Nos. 6 and 13) was conducted on DNA extracted from the same root segments used for GISH (Figure 9). M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder), GP corresponds to the barley cultivar 'Golden Promise,' CS refers to the wheat cultivar 'Chinese Spring,' and DW is the no-template control. The reactions were carried out using Phusion Green HF Buffer and Phusion Hot Start II High-Fidelity DNA Polymerase for optimal amplification conditions (Ali et al., 2024).

6.5 MPCR analysis of closely related *Triticum* and *Hordeum* species

To assess the broader applicability of the primers, we tested them on wild relatives and progenitor species of wheat and barley to understand their taxonomic limits. The *in silico* alignment of the final primer set with the available seven sequenced genomes (Suppl. Table S1: upper panel) showed perfect homologies and no unspecific products in the genome sequences of *T. spelta* (AABBDD sub-genomes), *T. turgidum* ssp. durum (AABB), *T. dicoccoides* (AABB), *Aegilops tauschii* (DD), and *H. spontaneum* (Suppl. Table S5). These results confirmed the high specificity of the primers for these species. However, alignment with the genomes of *T. urartu* (AA) and *H.*

marinum (XaXa) revealed the absence of several chromosome-specific target sites. Specifically, only four of the seven chromosome-specific target sites were found in *T. urartu*, and only two were present in *H. marinum*, with these sites marked in red (Suppl. Table S5).

In contrast to the predictions made from the *in silico* analysis, the MPCR results did not completely align with expectations for *Triticum* and *Hordeum* species (Figure 13). In *T. spelta*, a hexaploid species, the 7B chromosome-specific primer pair failed to amplify any product, a result also observed in the tetraploid *T. dicoccoides* (AABB), although successful amplification occurred in *T. dicoccum* (also AABB). Similarly, in *T. turgidum* ssp. durum (AAABB), the 6A chromosome-specific product was absent (Figure 13A: top panel). However, when these two problematic primer pairs were replaced with new ones (Suppl. Table S3), the correct products were successfully amplified (Figure 13B), showing that alternative primers can be an effective solution for these species. This adjustment highlights the versatility of the designed primer pools, which can serve as an additional resource for future applications.

The MPCR results with the A-genome species *T. monococcum* and *T. urartu* produced partial plex-A-specific patterns, with four and five products, respectively (Figure 13A: middle panel). Additionally, some unspecific products were generated with the plex-B primers. These results suggest that while the primers are generally applicable, some species may show partial amplification patterns due to genomic differences or primer-template mismatches.

Interestingly, in *Ae. speltoides*, the hypothesized donor of the B sub-genome, up to six correct bands were obtained with the plex-D primers, along with six faint bands from the plex-B primers and some correct-sized products from the plex-A primers as well (Figure 13A: middle panel). In contrast, *Ae. tauschii*, the definitive D-genome donor, also produced some bands with the plex-A and plex-B primers, further illustrating the genomic complexity of wheat and its wild relatives.

Finally, the plex-H primers, designed for barley chromosomes, worked as expected with *H. spontaneum* (HH genome), the closest relative of cultivated barley. However, when tested on more distant relatives such as *H. bulbosum* (HbHb) and *H. marianum*, the results were partial, with

fewer bands observed in these species (Figure 13A: bottom panel). This suggests that while the primers are effective for barley, their performance may vary across species with more distant evolutionary relationships.

In conclusion, the results demonstrate that the designed MPCR primer sets are broadly applicable across various wheat and barley species, but species-specific variations in primer performance underscore the need for flexibility and customization in certain cases. These primers serve as a useful tool for genetic analysis, offering reliable amplification for many species, but occasional adjustments may be required to optimize their performance for specific genomes or species.

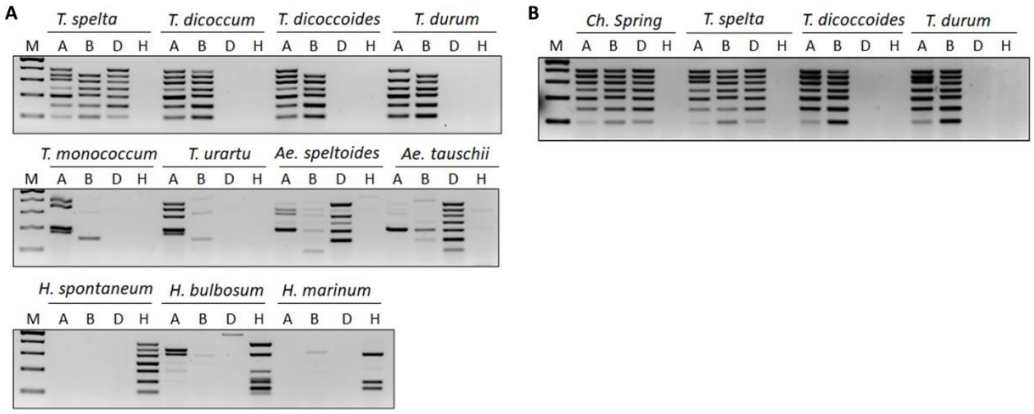

Figure 13: A. MPCR amplification of chromosomes 1–7 from the A, B, and D sub-genomes of wheat and the H genome of barley, using various wheat and barley species. **B.** Enhanced MPCR amplification with substituted primer pairs specific for the 6A and 7B chromosomes. M – Molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder). Reaction components: Phusion Green HF Buffer and Phusion Hot Start II High-Fidelity DNA Polymerase (Ali et al., 2024).

6.6 Transformation of barley with CRISPR/Cas9 vector

To justify our hypothesis of mutation creation in the wheat *MLO* gene via transgenic barley pollen, we identified the sequences of all three *MLO* homeoalleles (5A-TraesCS5A02G494700, 4D-TraesCS4D02G319000, and 4B-TraesCS4B02G322600) in 'Chinese Spring'. To obtain transformed barley lines, a designed sgRNA targeting all three homeoalleles was selected (Suppl. Figure S1). *Agrobacterium*-mediated transformations of 150 immature barley embryos with the pHUER vector construct containing Tamlosg2 (Suppl. Table S6) were carried out. Ten independent lines from three different calli were selected based on DsRED expression. For further confirmation, PCR was carried out on the hygromycin gene (*hptII*) (Suppl. Table S6) to verify the presence of the transgene. For crossing, only one plant from the T0 generation was selected and self-pollinated to produce the T1 generation. To check the heritability of the transgene, the selected T1 plants were further propagated to the T2 and T3 generations. It was found that in the selected representatives, the transgene did not show segregation.

6.7 Chromosome Composition and Mutation Analysis in F1 Hybrids

During the first crossing, we produced 37 F1 hybrid embryos. The embryos were examined under a light microscope at a wavelength of 550 nm to assess DsRED expression, and some embryos exhibited DsRED fluorescence (Figure 14E). Following DsRED expression analysis, the embryos were directly germinated on N6D medium. The regenerated plants were initially subjected to chromosome composition analysis using our MPCR-based chromosome composition detection marker system. All the F1 hybrids contained full wheat chromosomes, and the barley chromosomes were present in random numbers. Plants 2 to 12, along with 15, 16, 28, and 32, were identified as full hybrids. Plants 19, 24, 26, 27, 31, and 36 completely lacked barley chromosomes. Meanwhile, plants 1, 13, 14, 17, 18, 21, 22, 23, 25, 29, 30, 33, 34, 35, and 37 contained random numbers of barley chromosomes. Additionally, alongside H-genome detection primers, the Cas9 gene was amplified, as indicated by black arrows (Figure 14).

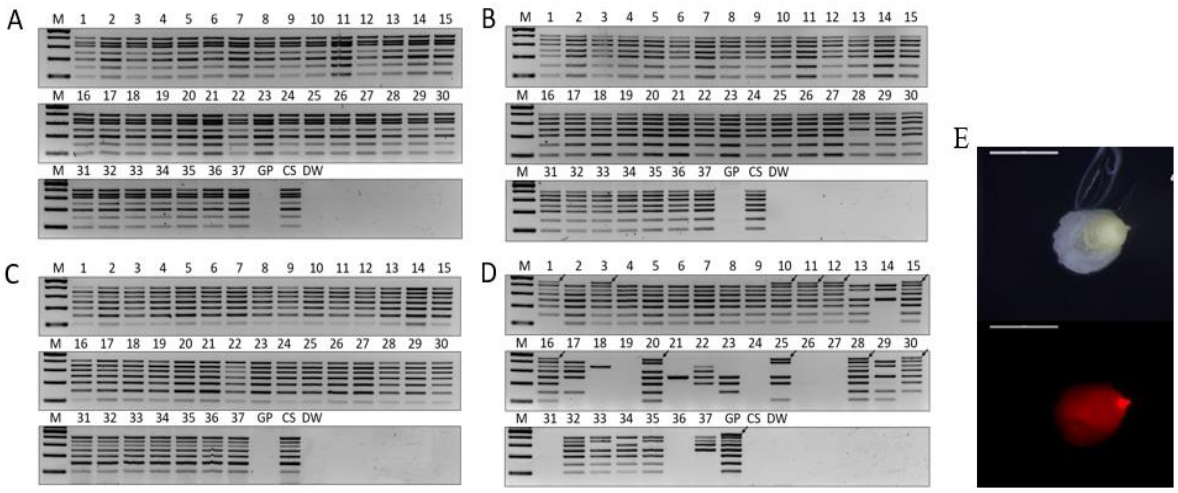

Figure 14: A. MPCR amplification of chromosomes 1–7 from the A sub-genome **B.** MPCR amplification of chromosomes 1–7 from the B sub-genome. **C.** MPCR amplification of chromosomes 1–7 from the D sub-genome. **D.** MPCR amplification of chromosomes 1–7 from the H genome. Cas9 positive plants are indicated with black arrows. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder), GP corresponds to the barley cultivar 'Golden Promise,' CS refers to the wheat cultivar 'Chinese Spring,' and DW is the no-template control. The reactions were carried out using Phusion Green HF Buffer and Phusion Hot Start II High-Fidelity DNA Polymerase. **E.** A hybrid embryo showing expression of DsRED under 550 nm wavelength (Lower picture) and same embryo at day light (Upper picture).

The PCR/RE assay shown in the image (Figure 15) was conducted to analyze mutations in the wheat *MLO* gene using specific primers, TaMLOABD_Seq_F and TaMLOABD_Seq_R (Suppl. Table S6), followed by digestion with the restriction enzyme *CaC*8I. Wild-type *MLO* alleles are expected to be fully digested, producing two distinct fragments, while mutant alleles lacking the *CaC*8I recognition site remain undigested. PCR amplicons from plants 10, 11, 15, 16, 20, 25, and 28 were not completely digested, suggesting the presence of mutations. Mutated plants are indicated with red arrows.

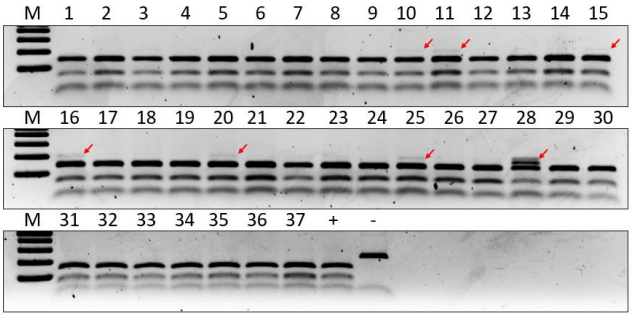

Figure 15. PCR/RE assay to detect mutation in F1 plants. *MLO* gene amplified using specific primers and digested with *CaC*8I restriction enzyme. Mutated plants are indicated with red arrows. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder). (+) sample is PCR amplicon from wild type 'Chinese Spring' with enzyme and (–) sample is WT without enzyme.

6.8 Chromosome Composition and Mutation Analysis in F1BC1 Hybrids

In an attempt at backcrossing all, F1 hybrids containing any barley chromosomes were backcrossed and successfully 16 backcrossed plants were produced. Most of the time one spike but sometimes 2 spikes per plant were backcrossed. Plants which did not have barley chromosomes were neglected. Like the F1 hybrids, these plants were first subjected to chromosome composition analysis using the MPCR-based chromosome detection marker system. All plants contained full wheat chromosomes. As with the F1 hybrids, plants 6, 7, 11, 12, 14, and 16 were identified as aneuploids with two sets of wheat chromosome from maternal and paternal side but barley chromosomes can be only inherited from maternal side, while plants 1 and 8 lacked all barley chromosomes. These plants were fertile but do not have mutation. Plant 13, which has only 4H chromosome, was also fertile but not mutated. The remaining plants contained barley chromosomes in random numbers. Plant 9 lacks the barley chromosome 5H entirely. Karyotyping analysis suggests it likely retains two copies of all wheat chromosomes, while possessing single copies of the remaining barley chromosomes (1H, 2H, 3H, 4H, 6H, and 7H). This plant was mutated but sterile. All other plants which have barley chromosomes were sterile and could not survive. Additionally, the Cas9 gene was amplified along with barley chromosomes, as indicated by black arrows (Figure 16).

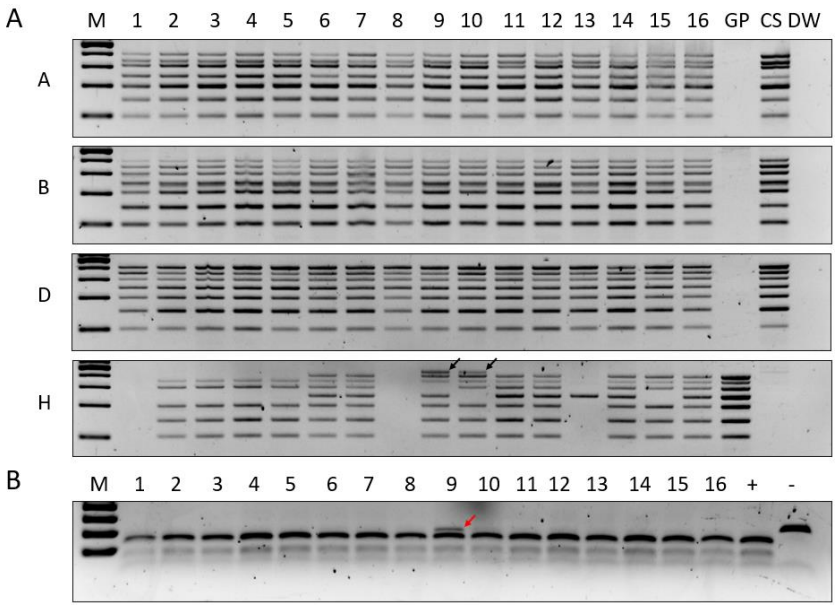

Figure 16: A. MPCR amplification of chromosomes 1–7 from the A, B, D sub-genome of wheat and H genome of barley. Cas9 positive plants are indicated with black arrows. **B.** PCR/RE assay to detect mutation in F1BC1 plants. *MLO* gene amplified using specific primers and digested with *CaC*8I restriction enzyme. Mutated plant is indicated with red arrow. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder), GP corresponds to the barley cultivar 'Golden Promise,' CS refers to the wheat cultivar 'Chinese Spring,' and DW is the no-template control. (+) sample is PCR amplicon from wild type 'Chinese Spring' with enzyme and (–) sample is WT without enzyme. The reactions were carried out using Phusion Green HF Buffer and Phusion Hot Start II High-Fidelity DNA Polymerase. The F1BC1 plants were derived from specific F1 parents and individual spikes. For example, F1BC1 sample 1 originated from F1 parent sample 3 and a single spike, denoted as (1:3, Spike 1). The complete list of F1BC1 sample origins is as follows: (2:2, Spike 1), (3:2, Spike 2), (4:4, Spike 1), (5:4, Spike 2), (6:5, Spike 1), (7:5, Spike 1), (8:6, Spike 1), (9:10, Spike 1), (10:12, Spike 1), (11:7, Spike 1), (12:7, Spike 2), (13:15, Spike 1), (14:8, Spike 1), (15:9, Spike 1), and (16:9, Spike 2).

6.9 *In vitro* propagation of F1 embryos

In continuous efforts of crossing where fewer F1 hybrid embryos are available, they can be propagated through callus induction. In one instance, we propagated 35 plants from one embryo. These plants were first subjected to chromosome composition analysis. As shown in (Figure 17), all 35 plants contained full wheat chromosomes for the A, B, and D sub-genomes. Regarding H chromosomes, the 6H chromosome of barley was missing from all plants, which can be expected

since all these plants were propagated from a single embryo. Additionally, in plants 28 and 31 along with 6H, the 1H and 2H chromosomes were also missing, respectively.

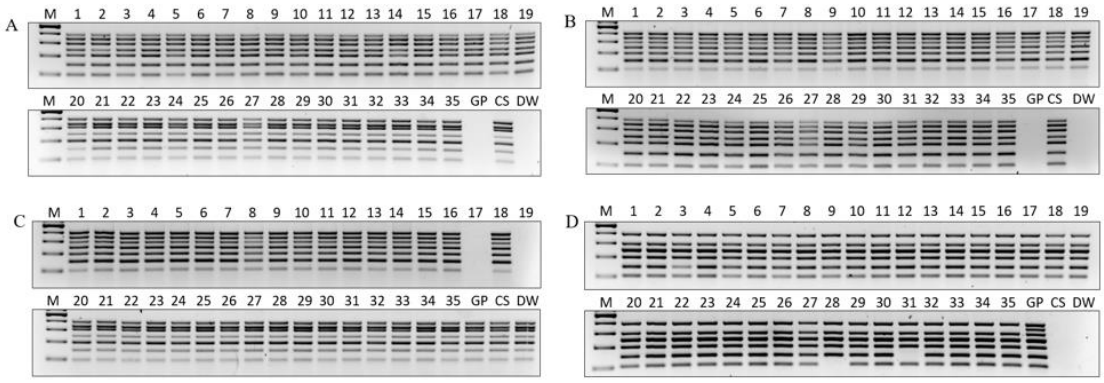

Figure 17: A. MPCR amplification of chromosomes 1–7 from the A sub-genome **B.** MPCR amplification of chromosomes 1–7 from the B sub-genome. **C.** MPCR amplification of chromosomes 1–7 from the D sub-genome. **D.** MPCR amplification of chromosomes 1–7 from the H genome. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder), GP corresponds to the barley cultivar 'Golden Promise,' CS refers to the wheat cultivar 'Chinese Spring,' and DW is the no-template control. The reactions were carried out using Phusion Green HF Buffer and Phusion Hot Start II High-Fidelity DNA Polymerase.

After chromosome composition analysis, mutation analysis was carried out using a PCR/RE assay. The *MLO* gene was amplified with specific primers - TaMLOABD_Seq_F and TaMLOABD_Seq_R (Suppl. Table S6). Following amplification, PCR products were digested with the *CaC*8I enzyme. Mutated plants are indicated with red arrows (Figure 18). PCR/RE assay results revealed varying levels of mutations among the analyzed plants, as indicated by the intensity of undigested PCR amplicons. While most plants exhibited low levels of mutation, as seen in faint undigested bands (e.g., Plants 13, 15, 18, 19, 22, 24 and 28), But plant 32 displayed significantly higher mutation level, evidenced by prominent undigested bands.

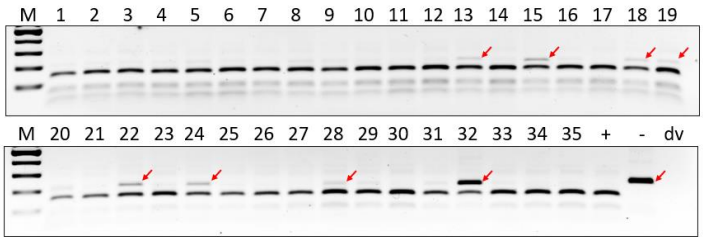

Figure 18. PCR/RE assay to detect mutation in F1 propagated plants. *MLO* gene amplified using specific primers and digested with *CaC*8I restriction enzyme. Mutated plants are indicated with red arrows. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder). (+) sample is PCR amplicon from wild type 'Chinese Spring' with enzyme and (–) sample is WT without enzyme. The reactions were carried out using Phusion Green HF Buffer and Phusion Hot Start II High-Fidelity DNA Polymerase.

6. 10 Cloning of F1 plants via immature inflorescence

To clone plant 32 from embryo propagation, which shows high degree of mutation, immature inflorescences approximately 1–2 cm in size was sterilized and placed on callus induction medium (Figure 19). Successfully, 28 plants were cloned. When these plants were analyzed for chromosomes composition, all these plants contain all 7 wheat chromosomes for A, B, D and H sub-genomes. And they retain the same barley chromosome composition. They have 1 to 7 chromosomes of barley except 6H chromosome was missing in all these plants. Which is expected because they are cloned from a single inflorescence. After chromosome composition analysis, the *MLO* gene was amplified with specific primers - TaMLOABD_Seq_F and TaMLOABD_Seq_R (Suppl. Table S6). Following amplification, PCR products were digested with the *CaC*8I enzyme. After analyzing these plants for mutations using the PCR/RE assay, most plants exhibited mutations with nearly identical intensities of digested fragments. However, some plants displayed varying levels of mutation, as evidenced by differences in the intensity of undigested PCR amplicons.

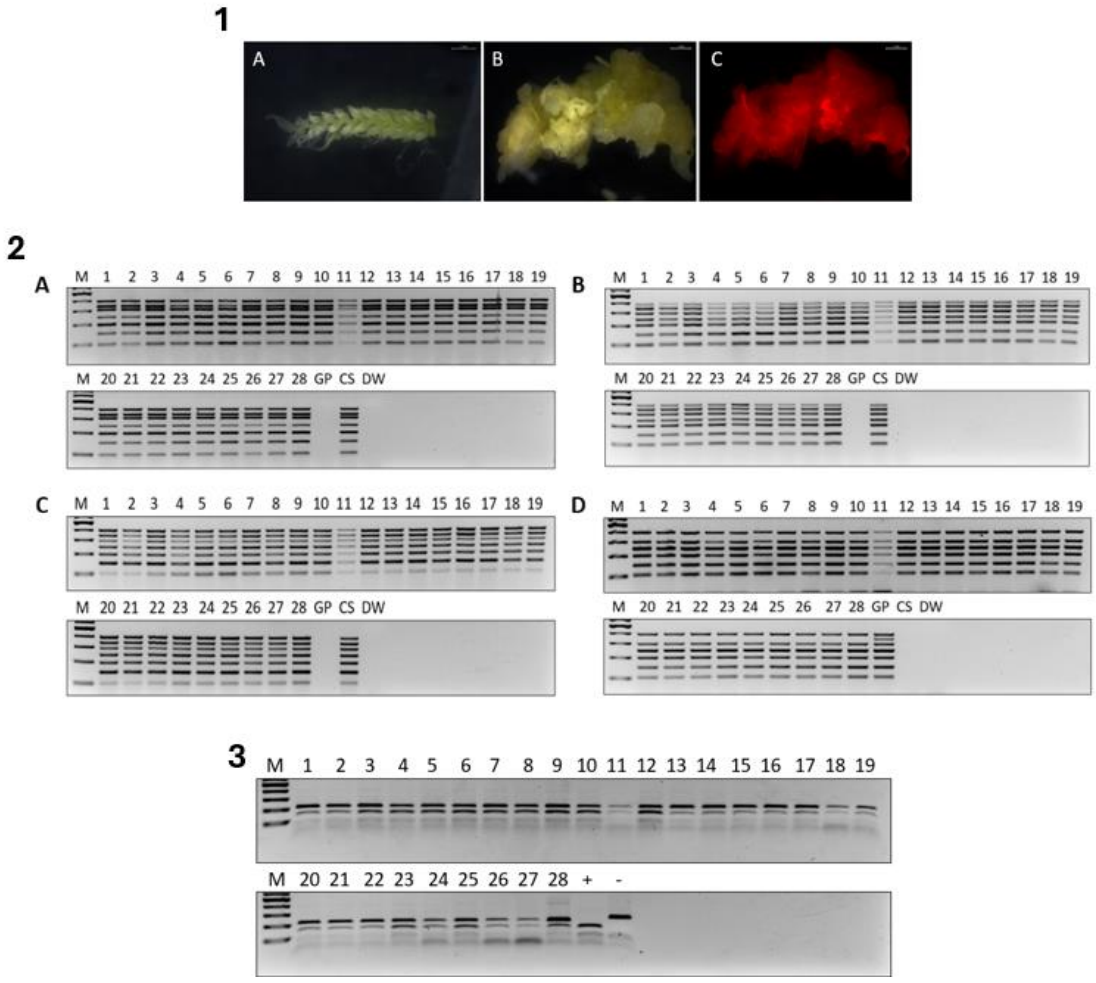

Figure 19. 1. Immature inflorescence tissue was placed on a callus induction medium to facilitate the generation of clonal plants. **A.** Immature inflorescence excised from the plant. **B.** Formation of yellowish callus tissue after successful induction. **C.** Fluorescent image showing DsRed expression in the callus, confirming the presence of the transgene in the induced tissue. **2. A.** MPCR amplification of chromosomes 1–7 from the A sub-genome **B.** MPCR amplification of chromosomes 1–7 from the B sub-genome. **C.** MPCR amplification of chromosomes 1–7 from the D sub-genome. **D.** MPCR amplification of chromosomes 1–7 from the H genome. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder), GP corresponds to the barley cultivar 'Golden Promise,' CS refers to the wheat cultivar 'Chinese Spring,' and DW is the no-template control. The reactions were carried out using Phusion Green HF Buffer and Phusion Hot Start II High-Fidelity DNA Polymerase. **3.** PCR/RE assay to detect mutation in immature inflorescence propagated plants. *MLO* gene amplified using specific primers and digested with *CaC*8I restriction enzyme. Sample 1 to 28 are PCR amplicons from cloned plants. M denotes the molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder). (+) sample is PCR amplicon from wild type 'Chinese Spring' with enzyme and (–) sample is WT without enzyme. The reactions were carried out using Phusion Green HF Buffer and Phusion Hot Start II High-Fidelity DNA Polymerase.

7. DISCUSSION

The development and application of a Multiplex PCR (MPCR) assay for the identification and tracking of individual wheat and barley chromosomes represent a major advancement in plant genetics, with significant implications for crop breeding, genetic research, and the characterization of hybrid plants. Wheat and barley are among the most important staple crops worldwide, and the ability to efficiently track specific chromosomes in these species is a critical tool for improving traits. In this study, we aimed to create an MPCR-based method for chromosome identification that could be applied in wheat × barley hybrids, offering a faster, more efficient, and cost-effective alternative to traditional cytogenetic methods.

Before delving into the specific details of the MPCR assay, it is crucial to understand the genetic relationship between wheat and barley, as well as their evolutionary backgrounds. Both wheat (*Triticum* spp.) and barley (*Hordeum* spp.) are members of the *Triticeae* tribe, and as such, they share a significant amount of genetic and evolutionary history. Wheat, in particular, is a highly complex polyploid species with multiple sets of chromosomes originating from different species. The hexaploid wheat (*Triticum aestivum*) genome, for example, consists of three sets of chromosomes from three different progenitor species, which belong to the A, B, and D sub-genomes. This polyploid nature of wheat makes chromosome tracking more challenging but also presents an opportunity to explore the functional relationships between different sub-genomes and species.

Barley, in contrast, is a diploid species (*Hordeum vulgare*) with one set of chromosomes designated as the H genome. Despite the difference in ploidy level between wheat and barley, these species still exhibit a considerable degree of genomic homology, with the general sequence homology between hexaploid wheat and barley estimated to be in the range of 45% to 60% (Bendich & McCarthy, 1970b; Flavell et al., 1977; Rimpau et al., 1978, 1980). This sequence similarity creates both opportunities and challenges for developing species-specific marker sets. On the one hand, homology allows for the design of primers that can distinguish between wheat

and barley chromosomes, but on the other hand, it also introduces the risk of off-target amplifications or cross-reactions, which could complicate the analysis of hybrid plants.

In order to develop the MPCR assay, it was crucial to select primer sets that were able to target species- and chromosome-specific loci in both wheat and barley, without producing nonspecific products due to the genetic overlap between these two species. The strategy involved the careful design and testing of primers specific for each chromosome in the wheat (A, B, and D sub-genomes) and barley (H genome) genomes. These primers were intended to be used in multiplex reactions, allowing for the simultaneous amplification of multiple chromosome-specific loci in a single PCR reaction, thus increasing the efficiency and throughput of the method.

One of the key challenges in this work was ensuring that the primers designed for MPCR would amplify the intended chromosome-specific targets without generating off-target products, particularly when applied to wheat × barley hybrids. The degree of sequence divergence between wheat and barley played a crucial role in the success of this approach. In the case of hexaploid wheat and its wild relatives, the polyphyletic nature of the wheat genome, resulting from multiple hybridization events between A, B, and D genome progenitors, led to some degree of sequence similarity across the sub-genomes. This overlap can complicate the design of primers that are specific to a particular chromosome.

Studies on the evolutionary history of hexaploid wheat have suggested that approximately 3 - 4 million years ago, hybridizations between the ancestors of the A- and B-genome lineages contributed to the development of the D-genome lineage, which would later give rise to the wheat D-genome donor species *Aegilops tauschii* (Zohary & Feldman, 1962). As a result, traces of sequence similarity are present among the A, B, and D genomes of wheat, even in the current wild species. This polyphyletic origin has complicated the development of precise chromosome-specific primers, especially for the D-genome chromosomes, and is reflected in the less precise chromosome identification observed in the wild relatives of wheat and barley (Huynh et al., 2019; Luo et al., 2017; Marcussen et al., 2014).

However, in the case of cultivated wheat and barley, the situation is somewhat different. The gene pool of these cultivated species is much less genetically diverse compared to the wild species, and whole-genome sequencing has provided comprehensive genetic maps for many of the common cultivars. This greatly facilitates the design of specific primer sets and enhances the accuracy of chromosome identification using MPCR. The availability of sequenced genomes for wheat and barley cultivars allows for the prediction of species- and chromosome-specific markers with a high degree of confidence, thereby improving the reliability of the MPCR assay.

The work described in this study also extended the application of the MPCR assay to wild relatives and progenitor species of wheat and barley, such as *Triticum urartu* (AA), *Triticum dicoccoides* (AABB), *Hordeum spontaneum* (HH), and *Hordeum marinum* (XaXa). These species represent important components of the evolutionary history of wheat and barley and are valuable resources for understanding the genetic diversity and evolutionary processes that have shaped the genomes of these crops.

The results from these wild species demonstrated some of the limitations of the MPCR approach. In some cases, the primer sets failed to amplify the expected chromosome-specific products, as observed in the case of *Triticum spelta* and *Triticum dicoccoides*. These discrepancies can be attributed to several factors, including the genetic diversity present within the wild species and the fact that some of the species tested may not have been fully represented by the reference genome sequences. Additionally, the polyploid nature of wheat, as well as the high level of genetic variation within the wild progenitors, may have contributed to the observed inconsistencies in the amplification patterns.

Despite these challenges, the MPCR assay was still able to provide valuable information about the chromosomal composition of these wild species, and the results suggest that further refinement of the primer sets, as well as the sequencing of additional accessions, could enhance the accuracy of chromosome identification in these species. In particular, the identification of chromosome-specific products in *Aegilops tauschii* and *Hordeum spontaneum* further confirms the potential of the MPCR approach for use in a broader range of species.

The traditional approach to chromosome identification in hybrid plants has relied heavily on cytogenetic techniques such as Genome *In Situ* Hybridization (GISH) and Fluorescence *In Situ* Hybridization (FISH). These techniques involve the use of fluorescent probes that bind to specific genomic regions, allowing researchers to visually distinguish individual chromosomes based on their unique sequence characteristics. While these methods are highly effective for chromosome identification, they are also labor-intensive and time-consuming, requiring extensive microscopy and manual analysis (Schwarzacher, 2016).

In contrast, MPCR is a faster and reliable throughput alternative to traditional cytogenetic methods. By amplifying specific chromosomal loci by PCR, MPCR can detect efficiently the presence or absence of individual chromosomes in hybrid plants. The method has robust advantages for F1 hybrids, where the chromosomes remain intact. In later generations, where crossing-over is possible, MPCR may be followed by cytological examination for verification. Additionally, MPCR is particularly useful in distant hybrid combinations where chromosomes do not recombine. This makes it an ideal tool for screening large numbers of hybrid plants in a short amount of time, significantly accelerating the karyotyping process. Moreover, the ability to use DNA extracted from various tissue types, including leaf and root samples, makes MPCR a more versatile and flexible tool compared to traditional cytogenetic approaches.

Additionally, MPCR can detect genetic mosaicism in hybrid plants. Genetic mosaicism refers to the phenomenon where different cells within the same organism contain different genetic makeups, particularly in hybrid plants. This is a common occurrence in interspecific hybrids, where cells may carry different chromosome compositions due to the random inheritance of chromosomes from the two parental species. Cytogenetic analysis may miss these mosaic variants, as only a limited number of cells are analyzed. MPCR, on the other hand, uses DNA from thousands of cells, effectively averaging out rare mosaic variants and providing a more comprehensive view of the plant's chromosomal composition (Koba et al., 1991; Taketa et al., 1995).

The Hi-Breeder study provides critical insights into the feasibility of employing CRISPR/Cas9-mediated genome editing in wheat through the use of transgenic barley pollen. By

designing a single sgRNA capable of targeting all three homeoalleles of the wheat *MLO* gene (5A, 4D, and 4B), we successfully created a robust framework for precise genetic modifications (Kis et al., 2024, WO2024224130). The transformation of barley embryos with the pHUER vector containing the CRISPR/Cas9 system and subsequent selection of transgenic lines confirmed the effective introduction of the transgene. The stability of the transgene across T0, T1, and T2 generations without segregation indicates homozygosity or multiple transgene copies, which are crucial for reliable delivery of genome-editing machinery during hybridization experiments.

The crossing of transgenic barley with wheat resulted in the generation of 37 F1 hybrid embryos. Chromosome composition analysis revealed significant variability in barley chromosome retention, with some hybrids retaining random sets of barley chromosomes and others losing them entirely. In this study, hybridization between wheat and barley resulted in 16.2% maternal haploids, 43.2% partial hybrids, and 40.5% full hybrids. While the frequency of partial and full hybrids falls within the range reported in earlier studies, the relatively low proportion of wheat haploids suggests reduced chromosome elimination efficiency, potentially influenced by genotype or culture conditions (Barclay, 1975; Koba et al., 1991; Koba & Shimada, 1992; Polgári et al., 2014, 2019)

Mutation analysis using PCR/RE assays demonstrated the ability of the CRISPR/Cas9 system to successfully induce mutations in the wheat *MLO* gene. The presence of undigested PCR amplicons in several hybrids confirmed the introduction of mutations, although the efficiency varied across plants. Some hybrids exhibited faint undigested bands, while others showed more prominent bands, indicating differences in mutation levels. These variations might be attributed to inconsistent Cas9 expression, differential sgRNA activity, or chromosomal context effects. Sometimes the Cas9 gene can be successfully amplified by PCR, yet no mutation is observed in the target plant. One possible explanation for this is that the target sequence was in a heterochromatin state, limiting CRISPR/Cas9 accessibility (Verkuijl & Rots, 2019).

Backcrossing experiments with F1 hybrids were carried out to stabilize the genetic background while preserving induced mutations. One of the significant challenges observed was the impact of barley chromosome retention on fertility and viability in hybrids. While hybrids

lacking barley chromosomes were fertile but did not exhibit mutations, those retaining barley chromosomes were often sterile. For example, Plant 9, which lacked the 5H chromosome and had all other barley chromosomes, carried mutations but was sterile. These findings suggest that the presence of barley chromosomes interferes with normal meiotic and developmental processes in wheat.

To address the issue of limited hybrid embryos, callus induction was utilized to propagate F1 hybrids, resulting in 35 plants derived from a single embryo. Chromosome composition analysis of these propagated plants revealed consistent loss of the 6H barley chromosome, with additional losses of 1H and 2H in specific individuals. Mutation analysis of these propagated plants revealed varying levels of mutations, with some plants exhibiting higher mutation intensities than others. This variability underscores the importance of optimizing *in vitro* propagation methods and Cas9/sgRNA design to maintain consistent mutation levels.

The successful cloning of F1 hybrid plants from immature inflorescences presents a crucial advancement in plant propagation. Plant 32, which exhibited a high degree of mutation from embryo propagation, was selected for cloning and cultured on a callus induction medium. This method resulted in the successful cloning of 28 plants. Chromosome composition analysis of these cloned plants revealed the retention of the same chromosomes across the A, B, D, and H sub-genomes.

To further validate genetic integrity and assess mutation presence, the *MLO* gene was amplified, and PCR products were digested with the CaC8I enzyme to determine mutation status. The results indicated that most cloned plants exhibited mutations with nearly identical intensities of digested fragments. However, slight variations were observed, which could be attributed to PCR amplification biases or template DNA concentration.

In conclusion, this study demonstrates the potential of CRISPR/Cas9-mediated genome editing in polyploid species through wheat × barley hybridization using transgenic barley plants. However, the variability in mutation efficiency, fertility issues, mosaicism, and uncontrolled chromosome elimination and retention in hybrids emphasizes the complexities of this approach.

While the MPCR assay proved effective for chromosome tracking, future studies should explore its application in additional crops like maize, rice, and dicots. The Hi-breeder study should investigate the functional outcomes of mutations through biotic resistance assays to fully validate this platform for genome editing in breeding programs.

8. CONCLUSION AND RECOMMENDATIONS

This study introduces a novel and optimized Multiplex PCR (MPCR) assay for the identification of individual chromosomes and monitoring their composition in wheat × barley hybrids, marking a significant advancement in plant genetics and breeding. By leveraging reference genome data from wheat and barley, large primer sets were designed to target specific chromosome regions. These primers were validated *in silico* by aligning them with genome sequences from 18 cultivars, confirming their specificity and reliability for chromosome identification. The experimental validation of these primers sets on 19 wheat and barley cultivars, along with 11 species from the genera *Triticum*, *Aegilops*, and *Hordeum*, further demonstrated their robustness. The successful application of the MPCR assay on 16 wheat × barley F1 hybrids, with results corroborated by the traditional GISH technique, validated the method's efficiency for chromosome-specific locus detection.

This approach has substantial potential for broader applications in crop breeding and genetic research, offering a scalable method for monitoring chromosome composition in hybrid plants. The primer design strategy can be extended to other plant species with well-characterized and sequenced genomes, making it adaptable for a wide range of genetic studies. The ability to quickly and accurately track chromosome composition will be instrumental in accelerating breeding programs aimed at developing plants with desirable traits, such as disease resistance, drought tolerance, and increased yield. For instance, wheat varieties that are better suited to specific agro-climatic conditions or possess resistance to pests can be developed. *Hordeum chilense*, a perennial diploid wild barley with significant potential for wheat improvement, has been utilized to create wheat-*H*. chilense chromosome $2H^{ch}$ introgression lines aimed at enhancing grain quality (Alvarez et al., 2019). Additionally, the MPCR assay offers a faster, high-throughput alternative to traditional cytogenetic methods, improving the efficiency of F1 hybrid characterization and karyotyping.

The use of Multiplex Polymerase Chain Reaction (MPCR) has emerged as a transformative tool in accelerating plant breeding by enabling precise and efficient identification of genetic

variations across complex plant genomes. Studies, such as Koh et al. (2017) for distinguishing Brassica species, highlight its application in polyploid crops, demonstrating cost-effectiveness and genome specificity (Koh et al., 2017). By optimizing primer sets for diverse genetic backgrounds, MPCR methods can enhance the sensitivity and robustness of assays, ultimately accelerating the development of crops with improved traits.

Moreover, integrating the MPCR assay with other advanced genomic techniques could significantly improve the accuracy of chromosome identification and provide deeper insights into chromosome structure and potential variations. Combining MPCR with technologies such as fluorescence *In situ* hybridization (FISH) could provide a more comprehensive understanding of chromosome integrity, translocation, and other structural rearrangements. These complementary technologies would allow for a more thorough genomic analysis, helping researchers detect subtle genetic changes that may not be fully captured by MPCR alone.

Sequencing efforts for wild species and progenitors of wheat, barley, and other crops are essential to enhancing the MPCR assay's reliability. By increasing the number of sequenced accessions, researchers will gain richer genomic data, leading to the development of more accurate and comprehensive primer sets. This will be particularly important for addressing the high genetic diversity found in wild species, improving the accuracy of the MPCR assay for species with less well-characterized genomes.

As genome sequencing becomes increasingly accessible, continuous refinement and validation of the MPCR primer sets will be essential to ensure the assay's adaptability and accuracy. Expanding the assay's application to a greater number of plant species and ensuring consistency across different cultivars will cement its place as a transformative tool in plant genetic research. The MPCR assay developed in this study represents a fast, cost-effective, and efficient method for analyzing chromosome composition and structure, with wide-ranging implications for plant breeding, hybrid characterization, and genetic studies.

On other hand the DNA free genome editing study represents a significant advancement in applying CRISPR/Cas9-mediated genome editing to wheat using transgenic barley pollen. By

targeting all three homeoalleles of the wheat *MLO* gene with a carefully designed sgRNA, we demonstrated the feasibility of inducing mutations in wheat through hybridization. The successful generation of F1 hybrids expressing the Cas9 system underscores the potential of barley pollen as a novel transgene delivery system for wheat genome editing. However, challenges such as the inconsistent and low mutation efficiency in some F1 hybrids, and reduced fertility in F1BC1 hybrids were observed. These limitations highlight the need for further optimization to fully exploit this innovative method. Despite these challenges, the study provides a robust proof of concept for the delivery of transgenic pollen having CRISPR/Cas9 system in wheat via wheat x barley hybridization to facilitate genetic improvement in polyploid crops like wheat.

To build on the findings of this study, several key recommendations are proposed. Increasing the mutation efficiency in hybrid plants remains a critical goal. Codon optimization of the Cas9 gene, designing more effective sgRNAs, or targeting less complex genomic loci could improve mutation rates across generations.

Hypothetically, the presence of unpaired (maternally derived) barley chromosomes in F1BC1 hybrid plants poses a challenge because it causes infertility. Although literature suggests that several additional lines exhibit disomic addition and remain fertile, it is also true that a full amphiploid has not been achieved despite numerous attempts. Future studies should focus on improving hybrid fertility, which is paramount for scaling this approach. Advanced mutation detection techniques, such as next-generation sequencing, should complement traditional PCR/RE assays to provide a more comprehensive understanding of editing outcomes. Finally, refining *in vitro* propagation techniques for F1 hybrid embryos can enhance the consistency of chromosome composition and mutation frequency, facilitating the large-scale generation of F1 edited plants. But propagation of F1 plants through immature inflorescence is another option to clone and get large number of edited plants. By addressing these recommendations, future research can unlock the full potential of CRISPR/Cas9-mediated genome editing in polyploid crops like wheat through transgenic barley pollen.

9. NEW SCIENTIFIC RESULTS

- A bioinformatics pipeline was designed to identify chromosome-specific primers, focusing on wheat and barley genomes, which can be applicable for other species
- A novel, fast, and cost-effective MPCR-based technology was developed to assess chromosome composition of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and their hybrids
- Additionally, the primer sets are compatible with various wheat and barley cultivars and effective on closely related *Triticum* and *Hordeum* species
- The MPCR methods is suitable for chromosome detection from any plant tissue, unlike the *in situ* techniques (GISH, FISH)
- Mutations were created in wheat *MLO* gene via wheat x barley hybridization.
- The barley genome carrying the transgene can be removed from the hybrid by backcrossing with wheat

10. SUMMARY

This thesis focuses on the development of a novel, fast, and cost-effective Multiplex PCR (MPCR)-based technology designed to determine the chromosome composition of wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and their hybrids. Hybridization between wheat and barley offers the potential to introduce valuable agronomic traits, such as resistance to biotic and abiotic stresses, into wheat varieties. However, the random chromosome composition of F1 hybrids necessitates a reliable and efficient method for chromosome identification and analysis. Traditional techniques, such as Genome *In Situ* Hybridization (GISH) and Fluorescence *In Situ* Hybridization (FISH), while highly informative, are time-consuming, labor-intensive, and require significant technical expertise, making them unsuitable for routine large-scale analysis. And on the other hand, they can be only performed on meristematic tissues such as root tips. This research aims to bridge this gap by introducing an innovative MPCR approach, offering a bulk and rapid alternative for chromosome analysis.

For this a bioinformatics pipeline was meticulously designed to identify unique 20-mer sequences from wheat (A, B, D genomes) and barley (H genome) reference genomes. These sequences were carefully filtered for uniqueness, GC content, and amplification efficiency. The pipeline included mapping primer pairs to specific chromosomes and optimizing them for MPCR applications. Primer pairs showing non-specific amplification were iteratively refined, ensuring high specificity across various wheat and barley cultivars and even closely related wild relatives (*Triticum* and *Hordeum* species).

Using these primers, wheat and barley chromosomes could be amplified, both in single PCR and Multiplex PCR formats. The MPCR assay demonstrated accuracy across diverse wheat and barley cultivars, their hybrids, and related species. Notably, the method was validated using GISH-FISH techniques, which confirmed the robustness and reliability of the MPCR assay in detecting chromosome compositions. Additionally, the MPCR technology proved effective in analyzing chromosome stability in wheat × barley hybrids, enabling the identification of chromosome retention and elimination.

The thesis also emphasizes the potential for applying the bioinformatics pipeline and MPCR methodology to other agriculturally important crops, especially where interspecific or intergeneric hybridization is used for trait improvement. This cross-applicability could significantly enhance breeding efficiency across multiple cereal crops.

In conclusion, this research presents a transformative advancement in plant genetics and hybrid breeding technologies. The MPCR-based chromosome analysis system is scalable, cost-effective, and high-throughput, addressing key limitations of traditional cytogenetic tools. The ability to rapidly and accurately determine chromosome compositions in wheat, barley, and their hybrids opens new avenues for advancing crop improvement programs.

On the other hand, the Hi-Breeder study explores the innovative use of transgenic barley pollen as a delivery system for CRISPR/Cas9-mediated genome editing in wheat. The research focused on inducing mutations in the wheat *MLO* gene, which is involved in susceptibility to powdery mildew, by targeting all three homeoalleles (5A, 4D, and 4B). Through *Agrobacterium*-mediated transformation, transgenic barley plants expressing Cas9 and an *MLO* gene specific sgRNA were successfully developed. These barley plants were used to fertilize wheat, resulting in the production of hybrid embryos expressing the transgenes.

The chromosome composition analysis of the F1 hybrids revealed varied retention of barley chromosomes, with full hybrids, partial hybrids, and plants lacking all barley chromosomes (maternal haploids). Mutation analysis using PCR/RE assays confirmed the presence of mutations in several F1 hybrids, although mutation efficiency was inconsistent. Subsequent backcrossing of F1 hybrids demonstrated challenges in maintaining fertility as most backcrossed plants with barley chromosomes were sterile, except one plant (plant 13) which was partially fertile having 4H barley chromosome. Additionally, *in vitro* propagation of hybrid embryos yielded multiple plants from a single embryo, but mutation inconsistency and sometimes barley chromosome elimination was observed during embryonic tissue development.

F1 plants also can be cloned from immature florescence via callus induction. When these plants were analyzed for chromosomes composition, all these plants contain all 7 wheat

chromosomes for A, B, D sub-genomes and they also retain the same barley chromosome composition. Which is expected because they are cloned from a single inflorescence. Following amplification, PCR products were analyzed for mutations using the PCR/RE assay, most plants exhibited mutations with nearly identical intensities of digested fragments. However, some plants displayed varying levels of mutation, as evidenced by differences in the intensity of undigested PCR amplicons.

The study highlights the potential of this novel approach for genome editing in polyploid crops, addressing challenges such as transgene retention, mutation efficiency, transgene delivery, and elite wheat varieties which are recalcitrant to transformation and tissue culturing. It provides a foundation for further refinement of this technique to improve its efficiency for functional crop improvement. This work opens new avenues for integrating DNA-free genome editing tools into the breeding programs of complex crop systems.

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12. PUBLICATIONS LIST

Conference Presentations and Posters:

Ali, M., Polgári, D., Sepsi, A., Kontra, L., Dalmadi, Á., Havelda, Z., Sági, L., & Kis, A. (2024, June 16–19). *Fast and effective MPCR-based molecular karyotyping in wheat, barley, and their cross-progeny* [Poster presentation]. EMBO Workshop: Plant Genome Stability and Change, Palacký University, Olomouc, Czech Republic.

Ali, M., Polgári, D., Sági, L., Havelda, Z., & Kis, A. (2023, November 30–December 1). *Establishment of powdery mildew resistance in wheat with CRISPR/Cas9 technology* [Oral presentation]. Genetics and Biotechnology Institute Days, Gödöllő, Hungary.

Ali, M., Polgári, D., Sepsi, A., Kontra, L., Sági, L., Dalmadi, Á., Havelda, Z., & Kis, A. (2023, May 15–20). *Rapid and cost-effective genotyping in wheat × barley hybrids by chromosome-specific multiplex PCR* [Oral presentation]. European Association for Research on Plant Breeding – Cereal Section Conference, Cereal Research Nonprofit Ltd. (GK), Szeged, Hungary.

Ali, M., Polgári, D., Kontra, L., Sági, L., Dalmadi, Á., Havelda, Z., & Kis, A. (2022, April 11–12). *A traceless CRISPR/Cas9 approach for precision mutagenesis in bread wheat (Triticum aestivum L.) via distant hybridization*. In *FIBOK 2022: 5th National Conference of Young Biotechnologists* (pp.86). Gödöllő, Hungary. ISBN 978-963-269-999-8.

Kis, A., Polgári, D., **Ali, M.,** Sági, L., & Havelda, Z. (2021, December 14). *Hi-Breeder: A new tool for efficient "DNA-free" wheat (Triticum aestivum L.) genome editing* [Oral presentation]. Genetics and Biotechnology Institute Days Conference, Gödöllő, Hungary.

Publications:

Ali, M., Polgári, D., Sepsi, A., Kontra, L., Dalmadi, Á., Havelda, Z., Sági, L., & Kis, A. (2024). Rapid and cost-effective molecular karyotyping in wheat, barley, and their cross-progeny by chromosome-specific multiplex PCR. *Plant methods*, *20*(1), 37. https://doi.org/10.1186/s13007-024-01162-x

Mihók, E., Polgári, D., Lenykó-Thegze, A., Makai, D., Fábián, A., **Ali, M**., Kis, A., Sepsi, A., & Sági, L. (2024). Plasticity of parental CENH3 incorporation into the centromeres in wheat × barley F1 hybrids. *Frontiers in Plant Science*, *15*, 1324817. https://doi.org/10.3389/fpls.2024.1324817

Polgári, D., Sepsi, A., Kis, A., **Ali, M**., & Sági, L. (2024) "Intergeneric wheat × barley (*Triticum aestivum* × *Hordeum vulgare*) hybrids: Production, Analysis and Deployment" *Methods in Molecular Biology*, (Accepted).

13. APPENDICES

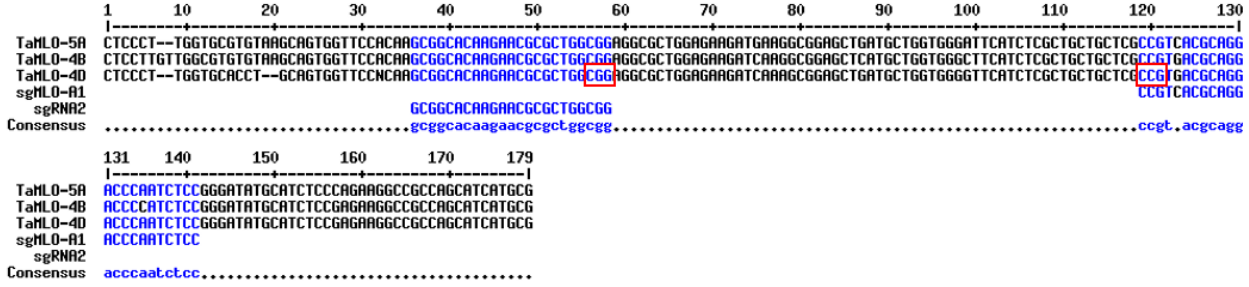

Figure S1. Sequence alignment of all three *MLO* homeoalleles (TraesCS5A02G494700, TraesCS4D02G319000, and TraesCS4B02G322600) was performed. The Tamlosg2 guide is also aligned with the sequence and shows a perfect match with the target site. The guide used in the study by Wang et al. (2014) is also aligned but has mismatches with *TaMLO-B* (TraesCS4B02G322600) and *TaMLO-D* (TraesCS4D02G319000). PAM is marked in red box.

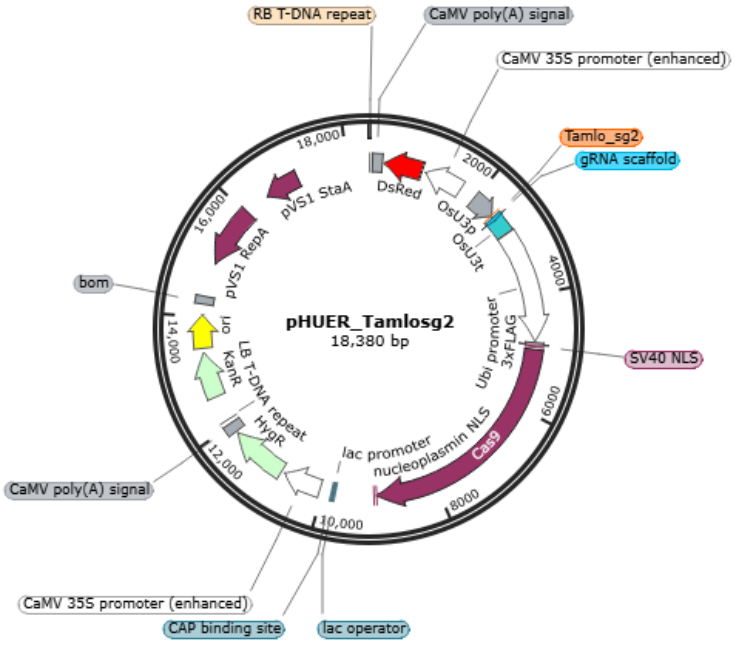

Figure S2. The plasmid pHUER_Tamlosg2 (18,380 bp) designed for CRISPR/Cas9-mediated genome editing in wheat. It contains a T-DNA region flanked by RB and LB borders, including the DsRed fluorescent marker for transformation detection and a hygromycin resistance gene (*HptII*) for plant selection. The Cas9 nuclease, driven by the Ubiquitin promoter, facilitates genome editing. Replication in bacterial hosts is enabled by the pVS1 RepA and StaA elements and a kanamycin resistance gene (*KanR*) for bacterial selection.

Table S1. Genotypes used for primer design, in silico analysis, and MPCR validation

Species	*In silico* analysis		
	Cultivar/Accession	**NCBI/GenBank ID**	**WGS Project/Assembly ID**
Triticum aestivum	Chinese Spring	GCA_900519105.1	IWGSC_refseqv1.0
Triticum aestivum	Fielder	GCA_907166925.1	CAJRHR01
Triticum aestivum	LRPB Lancer	GCA_903993975.1	CAJEVV01
Triticum aestivum	CDC Stanley	GCA_903994155.1	CAJEWR01
Triticum aestivum	Paragon	GCA_902810665.1	CADDYP01
Triticum aestivum	SY Mattis	GCA_903994185.1	CAJEWS01
Triticum aestivum	Julius	GCA_903994195.1	CAJEWQ01
Triticum aestivum	Cadenza	GCA_902810645.1	CADDYN01
Triticum aestivum	Weebill 1	GCA_902810675.1	CADDYO01
Triticum aestivum	Claire	GCA_902810655.1	CADDYM01
Triticum aestivum	Robigus	GCA_902810685.1	CADDYY01
Triticum aestivum	Jagger	GCA_903993795.1	CAJEVU01
Triticum aestivum	ArinaLrFor	GCA_903993985.1	CAJEVW01
Triticum aestivum	CDC Landmark	GCA_903995565.1	CAJFAH01
Triticum aestivum	Mace	GCA_903994175.1	CAJEWO01
Triticum aestivum	Norin 61	GCA_904066035.1	CAJFCQ01
Hordeum vulgare	Golden Promise	GCA_902500625.1	CABVVH01
Hordeum vulgare	Morex (v3, v2)	GCA_904849725.1	CAJHDD01, PRJEB34496
Triticum aestivum subsp. spelta	Spelt (PI190962)	GCA_903994165.1	Triticum_spelta.PGSBv2.0
Triticum turgidum subsp. durum	Svevo	GCA_900231445.1	Triticum_turgidum.Svevo.v1
Triticum turgidum subsp. dicoccoides	Zavitan	GCA_900184675.1	FXXJ01
Triticum urartu	G1812	GCA_000347455.1	AOTI01
Aegilops tauschii subsp. strangulata	AL8/78	GCA_002575655.1	NWVB01
Hordeum vulgare subsp. spontaneum	WB1 (AWCS276)	GCA_907165085.1	CAJRBJ01
Hordeum marinum	H559	GCA_022496015.1	JAAAWK01

MPCR analysis		
Name (lane no. in Fig. 4)	**Accession ID**	**Comment (source)**
Chinese Spring (1)	11113	gene bank Centre for Agric. Res.
Fielder (3)	W8354	W. Harwood (John Innes Centre)
LRPB Lancer (5)	10508	gene bank Centre for Agric. Res.
CDC Stanley (6)	19806	gene bank Centre for Agric. Res.
Paragon (7)	16469	gene bank Centre for Agric. Res.
SY Mattis (8)	16984	gene bank Centre for Agric. Res.
Julius (9)	16086	gene bank Centre for Agric. Res.
Cadenza (10)	11685	gene bank Centre for Agric. Res.
Weebill 1 (11)	20114	gene bank Centre for Agric. Res.
Claire (12)	15272	gene bank Centre for Agric. Res.
Robigus (13)	8009	gene bank Centre for Agric. Res.
Jagger (14)	6495	gene bank Centre for Agric. Res.
Bobwhite (2)	55767	gene bank Centre for Agric. Res.
Bánkúti 1201 (4)	18910	gene bank Centre for Agric. Res.
Igri (17) (H. vulgare)	17774	gene bank Centre for Agric. Res.
California Mariout (18)	117371	gene bank Centre for Agric. Res.
Golden Promise (15)	21877	gene bank Centre for Agric. Res.
Morex (16)	21031	gene bank Centre for Agric. Res.
Esperanza (19)	4945	gene bank Centre for Agric. Res.
Oberkulmer Rotkorn (spelt wheat)	MVGB1196	gene bank Centre for Agric. Res.
Mv Makaróni (durum wheat)	NA	gene bank Centre for Agric. Res.
Triticum turgidum subsp. dicoccum	MVGB135	gene bank Centre for Agric. Res.
Triticum turgidum subsp. dicoccoides	MVGB133	gene bank Centre for Agric. Res.
Triticum monococcum subsp. aegilopoides var. mayssuriani	MVGB100	gene bank Centre for Agric. Res.
Triticum urartu	MVGB108	gene bank Centre for Agric. Res.
Aegilops speltoides var. speltoides	MVGB1140	gene bank Centre for Agric. Res.
Aegilops tauschii	MVGB1324	gene bank Centre for Agric. Res.
Hordeum spontaneum 400154	MVGB1297	gene bank Centre for Agric. Res.
Hordeum bulbosum	RCAT042200	gene bank Centre for Agric. Res.
Hordeum marinum	MVGB1956	gene bank Centre for Agric. Res.

Table S2. Primers obtained by the refined protocol

	primer_Forward	primer_Revers	used in this study
1A-1	CCTCCTCTTCTCCCAGTTGC	GCCGGTCATACTGTGTCGGT	
1A-12	TTGTCGACAGGTCTCCGTGG	CCCAATGACCAGGGAGCTGA	
1A-18	GTGTTCCGGTAGTGGGTGGA	GAGAGGCAATCGGCTAGCAG	100bp_1A_F and R
1A-27	AAGCAAGGGCTGCCTCAAGG	AGCACCAACAGAGCAAGCCC	
1A-34	TATGGAACCTGCCAGGCCCA	GCGATCTCAACCCTAACCCG	
1A-46	GGCAGTCTCCGAGAAACGCA	CGAAAATGCTGGCCGTGCAG	
1A-83	TGCACGGTGCGAACTTCACG	CTGAGAGAGACCGGCACGAA	
2A-5	CGCAGCTCGCCACCGTTAAT	GACTGATTCGGAGCAGAGGC	
2A-10	TACTGGCCATGGCCATGACC	AACAGTGCAGTGGGTCCTGC	
2A-13	TACGAGGCTGAGGACGACCT	TGGAGTTCTCGCCGTTGCAG	
2A-20	CGCATCCAATGCAGGAACCG	AATCCAGAGCCGTGGAGCCT	
2A-23	TGCTTAGCGGCGCTCCTACA	TCCCTTGCCGCATCACACTC	
2A-32	TCTCACGTTCGCGTGTGACG	TGCTCGTCCGACGAACCATC	
2A-61	ACGTCTGAGCCCACTATCCC	GGGAGGTGTTGAGGAGGCAT	
2A-64	CCTCTGTCCCGGGATCTTGT	GGAGCACCGATCACGGAGAT	
2A-65	GGGCGGCCGTTGTCATACTT	TGGCGGTTCAGGCCCCAATT	
2A-70	CCATTCGCGCCTACGTTGTG	GGTAGGGATCGAGCTGGATC	
2A-79	ACCACCTGTCGTCCACCGAA	CCAACATCCGGTTAGGAGCC	
3A-16	GGCGCACCGCAGATGGAATT	GTCCTAAAGCGCAATGGCCC	
3A-42	GCCCCGGCGTGCAATCAAAA	CCCGTGCAGGTACCTGAAGA	
3A-47	GGGCTATGCCAAACCTCCAG	GAAAGGGGCCAATGGCCCAT	
3A-48	CGAGATAATCGTCTGCGGGG	GACCGGGATTGCTTGGCGAA	
3A-74	CCGTCACATCTCTTCTCGCC	CGGCAGGTAGCACAAAGCCA	
3A-77	CCCGCCCTTCGTAAGTACTG	TGACCGGGTTGCTGTGGTAC	
3A-94	ACCGTTGACCTTCAGCACCC	AGATGGGCCGAAACTGAGCG	
3A-98	AGCACACCTACGGCTGCTTC	GAGGATGCTATCGGTGCACG	
4A-9	CCAGCTCACATATGGGCGTC	ACGGACTCGGTGACGACGAT	
4A-19	TACAGGGAACCGCCATCGTC	CAAGTCACTTGGGCTGCCAG	
4A-36	GCACCCCTACGGGCATTTGT	TAGCGAACATGCGGAAGGGC	
4A-38	GAACAAGGTGCACCTTCCCC	ACGAGGAAGTTCTCCGCCGT	
4A-47	GGTCCTCGTCAGATCTAGCC	TAGCAGCGGCAGCAGGAACT	
4A-53	GGGCGCCCTACCCTTTGATT	CAACTGGCAGAGCAACGCCT	
4A-58	CAGCTGCCGCTTCGATCTCT	CTTGTACTGCTCCTCCTCCC	
4A-62	CCTCACTCTTGTCACCCTGC	CATCGCATTGGTCAGCCCTC	
4A-69	AGATGCGACTCCCCTGCGAT	TTTGAGAAATGGGCCGGGCC	
4A-77	GTGGTGCCTTGCCTTTCTCG	CAACCGCCTCCACTTTCCCA	
4A-80	CAATCTGGACCGGCACATGG	GGACCCCAAAAACCGATCCG	
4A-90	ACAAGGTGGCAAGCACCGAC	CTTGCATGGCCTCGGTACTC	
5A-8	GCTGGGCAGGCTCATTTCTG	GGCAAACCCTTGCCCCTACA	
5A-23	CGCATGCAGACCGAGCTACA	AATTGTACCGGGCTGGGCTG	
5A-29	CGGCAACGTTGATAGCGGCA	CACTTCTCAACCCATCCCGG	
5A-30	TTACCTCCGTTCTGACGGCC	AGGCGAGCATCAGCAAGGGT	
5A-48	GTGTCTCCTGGAGGGAGACT	GGAGGCAAAGAGGTCGAGCT	
5A-51	GCCACTATGGCACGCAATCC	GCTCCACCACTTCAGCTTGG	
5A-62	CCTTGCCTTGAGACTCGCGT	AGTTCTACGGGCCATTCCCG	
5A-72	GGACTCCCCAATCCCTTGTG	CCTGTGGGACCATGGGAGAA	
6A-3	ACAAGAGGATGGCCTCACGC	ACGAGCTGCATCAACTCCGG	
6A-5	CCCTCAAGTTCCGGTAGGTC	ACTGTCGGGGCCTTCTAGGT	
6A-6	GTCCATGGCACCAGGAGTCT	TCTGGTTCACAGGGACGTCG	
6A-8	AGGGAAAGCAGGGTGCGTTC	CGTGTCCCCAAGCAGCAACA	
6A-13	GTGCGGTCATGTTGCTGGTG	CTTGCCCATGCGAGCGTTTC	
6A-43	TGATGCCCCCCCTTCGACCAA	TCTCTGGCATAGGTGCTCGG	
6A-55	ACATGTACGGCGAGGATGGC	TGACTGAAGAAGAGCCCCCC	
6A-66	TAGCCAGGTTTGCAGCGAGG	TGCTTCGATTGCGGACTGCG	
6A-69	GTGGTACGTGCAGCGGTTAC	CGGGACCAATCTAGGAGCCT	
6A-77	GAGGGCGCCAACTTTTGGAG	ATCGAGTCGGCGGAAATCGG	
6A-88	TGATGGGAGGTGGAAGGAGG	TTTTAGCACCCGGGGGCCTA	
6A-93	TGGCCTCTGCGTGGACTATG	CCCGGATTTGTGGATGCACC	
6A-98	ACGGGTCAACGCAGACTCGA	AGCGCCTCGATGAGAACCTC	
6A-99	AATGGTTGTGAGCCGCCTCG	AATCCGGCGGCTTGGCAGAA	350bp_6A_F2 and R2
7A-15	AACGGTCGCCTGGTACTCGT	GTGTCACCGGTCTTCCTCCT	
7A-47	GTCCGGACGACTTCCACTTG	GAAGCGAGATCTCCGCATGC	
7A-67	AACCGGCACAATCGAGCGTC	CTCTACCCCTCTCGTCGCTT	
7A-79	GATAGGGGGAGATCAGGGTG	GGTCGTCTACCCAGCTCTAC	
7A-80	GGGGAAGCCCCTGGAAACTT	GGGGACGACAATAAGGGGCA	
7A-99	GTTGAGCACCGTCAGTGTGG	AGGGGAGGAACGGATCTGAC	
1B-38	CCACCAGGGGAAGACAGACT	GGCGAATGGAGCCAGTGTGA	
1B-41	ACAGTTCGCGCTCATCCGCT	GTGCACGACATCTTCCCAGC	
1B-61	GGCGGCAGTGAGAACGAGAA	CGCCGTCACCACTGCTATAG	
1B-70	GAGCAGGGGAGGACTAGTCT	TGCGACCTTCCTCGAAGCTC	
1B-74	CGCGCAGTCATATTGCACGG	ATACATGGACGTGGGCCAGC	
1B-89	AATTACCGCCTCAGGCCAGC	GCTCAAATGGAGGCCAGACC	
1B-98	GAGCACAAGCCACTGCCACT	GGACTGAGCAAAACCTCCCG	
2B-30	TGGGCTGGGCATCCCTCATA	TCGGAGCGATGAGCTGGATG	
2B-73	TGGCCCTTTGGCTGGTGCTT	ACTATGTGCCGCTGATCGCC	150bp_2B_F and R

2B-79	AGAATCCGGGGGCTTCACGA	TAGGAATGCGCGGCGATGCT	
3B-4	CCTATCTATCTCCCCAGGGC	CCTGCATCAGTAAGCCAGCC	
3B-18	CCCGCCTATAGTACGCGTCA	AGACGGGTCTTTCACTCCCG	
3B-73	GCTTCTGTCGACGGGAGAGA	GCTTCTTTCACGAGACGCGC	
3B-98	CAGGACTCCCATCTCGATCC	GGCTTCCTCCGATCTGTGCA	
4B-3	ACGTGTGGACTCTGGAGCCT	TTGGTTGGTGCCATGGTGGC	
4B-13	TCGGTGGTCTAGGCCATGCA	AAGCCCGGTAGCAGATCCAG	
4B-40	AACGCCGACCCCAACGCAAA	GGCTGAAAACGAGCCCATGG	
4B-77	ATCCCGAAGCTCTAGACCGG	TGTCGGTGACCTTCCTCCCT	
5B-4	GGGGCTTGGGATTAACTCGC	GCCTTCTCGGTGAAGCTTCC	
5B-8	GTTTAGCTGCTGCCTGCGAC	ATGCGAAACTTCCTCCCGGC	
5B-18	GGACGCTGGTTTCAGCATGC	TTCGTGCCGGTCCTTCCTTC	
5B-24	TCAGAGATGAAGCGGCCGAC	GTCTTCACCAGCCTGAGAGC	
5B-32	GGTGTTAGCCCGTGCGGTTT	AATTTTGGCTCCCGCGCCGA	
5B-44	CAGGGCCACCGTTTCCAGAA	TGTCATGGGAAGGCAGGGTG	
5B-57	CCGACTCAAGGAAGGACCTC	CCTGATCACGTCCATGACCG	
6B-10	GGCCGATCTTGTCCTGAGAC	AGCTTTACCCCGCAGCTTCC	
6B-11	GAGGGGGAGTTACTGCACCT	CTACCGACTCGGCTCCTTAG	
6B-46	GCTGGGGTAGAGCCATTGGT	GCCAAAGCCTCGATGGGAGA	
6B-47	GCACGCTTGAGACACCGTAC	TCCCGTTCCTTGTTCCCACC	
6B-52	AGAGAGTAGGCACGGCGTAG	AGTTCCCTCAGGGGGAGACT	
6B-56	GCGGTGCTCGATTAGGGTCT	GTAAGGACATCTCCGACGCG	
6B-66	CTACTACTCAGCACAGGCGC	AGGTACTTCACCACCGGCCT	
6B-79	CGTCCACATCCCGACCAATC	CAGTTGGGCCCAACTGCTTC	
6B-82	GCAACCGGTGCCAACTCGAT	GGATCGGGGATCGAGTGCAA	
7B-8	GCACCAAACGGACCACCTGT	AACGATGGCGACGGTTGTGG	
7B-10	GTGGATCGAGACAGTCCTGG	CTGCGATCCGAGTGGCTACA	
7B-11	AACACGCATCACCGACGTCG	ATTGGGGCTGAGGTCGAGGT	
7B-30	ATCATAGACCGCCCTGCGTC	GGAGGACTTCCGAACAAGCC	
7B-38	ACTCAGTACTCGGCCTTCGC	CCGTCATCGACGCGGTAGAA	
7B-55	AGAGCGTAGAAGCGCAGCTC	TACGGCGATGGGAGCTAGCT	
7B-57	CCCCCTGTCGCTGATCCAAT	ACCCGACATTTCCCTTCCCC	
7B-63	CTTCGATCGGGAAGGTCGTC	TTCCCTCGTTCCTACCCCGT	
7B-64	GGCCCACTCAATCGACCGTT	TGGCCGGGAGCAAAACCCTA	
7B-72	GGCGACGGATGTGGCAACAT	GAGCTCCCTGGATTCAGAGC	
7B-79	CTCTGGACGTCAGCCTTAGC	CGTGAGGTCGCATAGAGTGG	400bp_7B_F and R
7B-91	GCTAGGGGCGTAACCGTACT	AGTGGCTGCATTGGCGCAGT	400bp_7B_F2 and R2
7B-96	CATGGCTCGATGGATGAGGG	ATCGCCTCTAAAGGCGACGG	
1D-7	CTGCCGAGTCTAGCCATGAC	ACCTGGCCTGCCATTCAGAG	
1D-27	ATCCAGGTAGGGAGGTGACG	CGGCAGCATCCATCCGATGT	
1D-39	CACGGCCGTCCTAATTCCTC	TCCTCATCCATCCTTCCCGC	
1D-43	AAGCTGCAGCTATGGCGGGA	AACCACCACAGCTGGGAACC	
1D-47	TGTGGGTGGGTTGCTTCTGC	GGGTAGACCAGCGTCAATCC	
1D-66	AGGAATCTTGGCGCAGGGAG	TGGGAGGAGGAACGACCTAG	
1D-86	ACCTGCCAGTCGCCACACTA	GTTGAAGTTGGCCTTCGGCC	
1D-99	AACCACCCCTGGTCACGTGA	CTTGGAGCAAGGCCTTAGCC	
2D-38	GCCCTCCCCCCTTATTTTGC	TGGATCCTGGATGGATGGCG	
2D-47	GTTGAGGGGGCTCAGATAGG	ATGCGGACGCGGCTTCCTAA	
2D-87	AAGCACATGGATCGGGGCCT	CTCCACTACGGGTCTCGCTA	150bp_2D_F and R
3D-5	TAGCCGGGCAGACCGAAGTT	TCATGCCGAGCATGCCATCC	
3D-9	GCTTCGTATGGCCATCCGTC	CATCATCCCTTCACCGCCGT	
3D-12	ATCAGCCACGGCGAGTCCTT	CACAGATAGCGCGATCGACC	
3D-13	AGGAGTCGTTTTCCCAGCGC	CGCGCAAGTAGAGAGATGCC	
3D-19	CGGCCGATCGATGACCACAT	GCATCGCCATGGCCAATGCT	
3D-20	ACCTGCTATTTCGGCCCTGG	GCCACACCCTGAAGTCTGAC	
3D-60	ACGGCTGCAGTCCGATTCAC	ACTAGGCGCAGGTTCGTCGA	
3D-63	CTAGCCCCGATCCAGAGATG	GGTCGCCAAAGCGTTCAGAG	
3D-74	GATGTCGCCGCTTCTCTAGC	GAGGAGTCCATCTGGGAGAG	
3D-75	TCGTACTCCAGATCCGCTCC	CCCTGCTTCCAAATCCGACG	
3D-83	AAGTTCGACCAGGGCGCATG	CTCCACTAGCCCCTAGGGAA	
3D-85	CATTAGCACCGCTGGTGTGG	GACGGCAACGAGGATGATCC	
3D-88	GCGGGGTGACGCTTTAGATG	AAACTGGCACTGGGCTGGGT	
3D-99	GACACCACGACGGCTGTCTT	CAGTGAGCTCTGGAGCAGCA	
4D-12	GCAGCCCAAAAAGTGTCGGG	CCCTGAACGCTTGCAGTTGC	
4D-18	TGCTTTCCGGCAGCCCAATC	CATCGCGGACAGGGAGATGT	
4D-19	GAACAGCACCATAGGGCACC	GGGTTCGCGCCTTCTTGATC	
4D-31	ATAAGAGAAGGGCCCCGAGC	GACAAGGTCCCTCGTGTACG	
4D-32	CCGGACCTATTCGTACGCGA	TCTGCCTGCCATGTTGCGGT	
4D-48	GTATCGGGGAAAATCGCGCC	GATAGGAAGGGGTGTGCCCT	
4D-53	CCCTCTCTCGCTCGATTCCT	CGAACCTCATAGCGCCGAAG	
4D-60	ACGACCTGCCTGACGTCTTG	TCCCACCCTCAAGCGAGAAC	
4D-75	GTCGCACACTTGCCTAGCTG	AGCAGAGGAAGCATCCAGGG	
4D-87	ACTCCGGAAGCGGAGTTGTG	GACTAGGCACCTAGGCATCC	
5D-15	GCAAACTCTCCCCCGATCGT	AGACGGGCTGAAGTTCCTGC	
5D-18	GTGTTTCCTGAGCTGGCACC	TGCGGTGGTTCCTAGCTGCA	
5D-21	GTCGGATCCTTCAGTGGCTG	GCCCATGGATCCATGGTAGC	
5D-30	CGTCATTCACCGGCACTGGA	CATCCCCAACCTACTCGTCG	

5D-38	GGGTAGAATGGGCTAGCCCA	ATGGCTGCAGCCGAAACCCT	
5D-41	TTCTGCAGGAGCAGCAGCGT	TGCTTCATCGCTCCCTGCAG	
5D-62	GGGGTCTTGCGGCTGAAATC	ACCAGACGCGAGACAAGCGA	
5D-73	GAGCCACGCAGAGTCTTGAC	TTCCTTGTCGCCGGTCTGGT	
5D-77	AGCGCCTTTGGATCTAGGCC	GACCTCTATCCCGCACTCCT	
5D-85	GTAACTGCTACTCCTGGCCC	CAAATCAGCCTCTGCCTCCC	
5D-87	CGTGATCGTCGCGTTTTGGG	GCATGGCAGCTGGTTGCTTC	
5D-91	ATGTGAAGCGGCGGAGGAGT	GACGATGTACTACTGCCCGC	
5D-96	GCGGTCAATGCCTCGTGCAT	ACTAGAGCGTGGATGAGCGG	
6D-23	GACAGGCTGCACGTCTGAAC	TCGCCGCGCAGCACATGTTT	
6D-40	AGAAGGAGACGAAGCGGCGT	GACGGACGGACGTCTTGATC	
6D-48	GCCTGACACAGGGCCAACTA	GGGTTCCAGTTCCACGCCTT	
6D-49	AAAGGAGCCTCTCCGCCAGT	AGGCTGGTCCTCTCCTTCTC	
6D-66	CCAGGGCCCAATGATGAGAC	GATGACCCAGGAGCCCACTT	
6D-81	CATCGATGAGGGGGGACAAC	GTGTTATCACCCGTCGTCGG	
6D-94	GCGGGAATATGCCTGGAGTC	TTTCACCCGGGGAGTGAGAG	
6D-100	TTGGCAGACCAGGTCCTAGG	TTGCGCCTTCCCAAACTGCC	
7D-7	TATCACGCGCTGGCTCGGTT	AGAGGAACACCAGGAACGGC	
7D-9	TTGGGGCCAACTTGTCCCGA	GCACGATGGAGAGGATCGAG	
7D-14	TGACGCGTGAGCTCGATGGA	GAGTTGCGCGCGCTGCATTT	
7D-16	GCTCCTCCGACAGAATAGCC	GAGTGAAGAACGGAGCAGCG	
7D-23	AGGCGAGTTACGCCGTAGTG	CCTCCGTAGCCAAACCATGC	
7D-28	CCAGGTCTGGTCTGCACAGA	GAGTCGCTGACCATGCCACT	
7D-30	GGCCAGTGGTCCATGAGCAA	TTTCTTCGTTGTGCGCCGGC	
7D-54	CAGAAGTGCACCTGGCGGTA	TTTGCCAGCGGTTGTACCGC	
7D-55	CGTACGCTAGGTCTGGATCC	CTCCACCTGAATGGCCCGAT	400bp_7D_F and R
7D-56	AACTACCCCACAACCGCACG	GGTGCCCTTCTCCACCTTTG	
7D-71	GATGGAGTCACAGCGCAGGA	AACCTAAGGACTCGTCCGGG	
7D-72	GCAATGGGCGGGGCATCATA	CACCTAATCCAACGGCCCAG	
1H-5	ACGAAGTAGCCTGGCTCCCA	ATGGCGGTCGTCTCTCTCTC	
1H-31	AACGGCGATGGAGAGGAGTG	CTTGTCCTCTCCGCTGTCAC	
1H-58	GTTGACTACCGCTGCAGCAC	GGCCGATGTTGTGGACATGC	
1H-74	GTCGGTCGCGATTTTCGTGG	TCGTTGTGGCAGGAAGTCCC	
1H-87	TCCACTAGACGCAGCCTAGG	CCTTGTTCTCCTGCTGGCAG	
1H-89	AAGAAGGACGGGCAGTGTCC	GTCCAGCTGCATCTGCAGGT	100bp_1H_F and R
2H-10	CACCGCCAACAAGGCCTGTA	CCCACCTGATTACGGTGGCA	
2H-14	AAATAGCGGCGCACAGGGCT	CTTCCGGTGTCGGCGTTGAT	
2H-17	ATGAGGGGGTGTGTGGTCGA	TGCACCTCGAGCTCGATCCA	
2H-31	CGCGTCGATCGTGTCCATAC	GCGTGCAGGCCTAATCCATC	150bp_2H_F and R
2H-43	CCAGGGATGCGTTTCCTCTC	GGGAAAGGAGGGGAGGTCTA	
2H-59	TGTGTGCCTCGTAGTGAGGG	CACTGGGCTGAGGTGCTCTA	
2H-62	ATTACGCCCACGACCCAACC	CCACTTTCTGCACGACGGGA	
2H-72	ATCCTTGAGGCACTCACGGC	CTGCCATGGGAGGAGGATTG	
2H-73	TCGAGGGCATGGTAGTTGGG	AGCATAGGACGAACCCCAGC	
2H-77	AAGCCTGGTCCCAACGACAG	CATCCGTTGGGAGAAGCTGC	
2H-93	CTTCTTGCTACTCGCGGAGG	TGGGACATGCAGTCCTTGCC	
3H-31	GCTGCATACGCTCCCCCTTT	GTCAGACGATCTCGTTCCGG	
3H-40	GCTGAAGGCGTATGGGTCGA	AAGGTTCCTTCTCCCGTGCG	
3H-63	TTCTCCAGCCGTGCACACAG	TCCAAGCCGTTTCCTCCAGC	
3H-64	GGAAAGCCTTGTCTGCCGTC	CTCAGTGTACTCGTCACGGG	
3H-70	TGGCAATAGGGGCATCACCC	CCGAATGATCGGGGTTCCAC	
3H-74	GGTATCACCTACGTGGGCCA	TCCTTCCTCGATCCAGCAGG	
3H-75	AGCAACTGGCACCGGCAGAT	TGTGCTTGCCCTGCTTGCTG	
4H-6	GGGGCCGTTATCCAGGACAT	TCACTCTCACTCTGCGGTGG	
4H-13	CTCGACAACCTCTTCCCCTC	AGCGCTAACCTCTTGCCCTC	
4H-22	GGCGGTGTATGTGAGACCGA	TTCATGTCCGAGCCGGACTC	
4H-78	CAAGAGGGCTCATCGGTGAG	TGGTCTTCTGCAGTCTCCCG	
4H-85	AAGGTATCGTCCCCCTTCGC	TCCAGGTCCTGGCCGTTCAT	
4H-93	CCTGAGTCTGTTGGCTCACC	CCGTGTGAGTTGCTGCTTGG	
5H-18	AAGACGATGGGATGTGCCCG	AACGGCGGGCGAAGAAGATG	
5H-25	GGTACTCCATGAAGGGTCCG	CACTTGTGGGCGATGGATCG	
5H-50	CTCTCTCTGTGGGCGCTAAC	TAGGTGGGTCGATCCGAGGA	300bp_5H_F and R
5H-83	GTAAGCGATGGCGGGGTTCT	AGCTTGGACCCTGGGAAGAG	
5H-86	ATCTCGGACCTTCAGTGGCC	GCGAAAGAGGAACTGGGCGA	
6H-23	TCCCCGACCACATCCTTTCC	TCACCTTTGGGGGTTGGTCG	
6H-34	AATGACATCCCCGGGGGTTG	AAGAACCTCCTCCCGGGCTA	
6H-46	TACAGCCCCTTGTCGATGGG	AGGCGCGCTCATGATCAGTG	
6H-82	TCTCTGAGGAGCTGGAAGGG	TCCTGCCTGATTCTCCGGCT	
6H-84	GCCATGCAAGGGCAACTAGC	CTCCCGGAGATCTGATCCGT	
7H-10	ACTGCGTGCATCCTCTGCCT	CCACTGGACACATTTGCGCG	
7H-13	CTTGGTAGGGAGCTACGCCA	CCACCATTCTGCTAGGGGCT	
7H-38	TCTGCACTCAAGGTGTCCGC	GGCATACGTCAAGCGCTCGA	
7H-40	CCACCGGCGTTTCTTGTTGG	CTGCTCCATTGCTCGGCAGT	
7H-46	GGGTGACGGAGTTGCATCGA	TGAATGGGGCCACCCACGAT	
7H-64	TGGGGAAGACGCGCTATCGT	GAGAGGAGGCGCAAGTTCCT	
7H-77	CGACGAGGAACGACCGACAT	CGCACACACACCTTATCCCC	
7H-99	GCCCCGGGTCGAAAGGAATT	GCTGCTTCTGAGAGGATGGG	

Table S3. The final list of chromosome-specific MPCR primers in the A, B, D, and H (sub-) genomes

Primer name	Sequence (5'-3')	Length (nt)	Tm (℃)	Genomic location (on reference genome)	Product size (bp)	Comment
100bp_1A_F	GTGTTCCGGTAGTGGGTGGA	20	59.1	1A:538121084-538121103	100	
100bp_1A_R	GAGAGGCAATCGGCTAGCAG	20	57.9	1A:538121164-538121183		
150bp_2A_F	TGCCGGCCATCAGTTGATGG	20	60.6	2A:722058206-722058225	150	
150bp_2A_R	GTCTCTATGGCGTACCGGAC	20	57.0	2A:722058336-722058355		
200bp_3A_F	CACCACCTCGATGGATACGC	20	57.9	3A:381302864-381302883	200	
200bp_3A_R	CACACGCTTGGTCTAAGGCC	20	58.6	3A:381303044-381303063		
250bp_4A_F	AGTACAGCGAACCTGGCAGC	20	60.1	4A:193096666-193096685	250	
250bp_4A_R	CGCTGCTAGTTCAAGCAGCC	20	59.1	4A:193096896-193096915		
300bp_5A_F	AGGCTCACTCCAACACGACC	20	59.6	5A:32789504-32789523	300	
300bp_5A_R	GAACAGAGTGTGGCGAGAGG	20	57.7	5A:32789784-32789803		
350bp_6A_F	TGCCGAGATTGGACGTCACG	20	59.9	6A:605537994-605538013	350	
350bp_6A_R	GTGGTCTGTTGGTGGCCTTC	20	58.6	6A:605538324-605538343		
400bp_7A_F	TGGCGGCCATTAGACTAGCG	20	59.7	7A:433054330-433054349	400	
400bp_7A_R	GGCGTCTCAGGTGTTGGACT	20	59.6	7A:433054710-433054729		
100bp_1B_F	CGTACCACTATCACGGCAGC	20	58.0	1B:102525056-102525075	100	
100bp_1B_R	CTGGCATGAAGCCGTGTGCT	20	60.9	1B:102525136-102525155		
150bp_2B_F	TGGCCCTTTGGCTGGTGCTT	20	62.8	2B:649462075-649462094	150	
150bp_2B_R	ACTATGTGCCGCTGATCGCC	20	60.2	2B:649462205-649462224		
200bp_3B_F	CGCTTCAACCCCGCCTATAGTA	22	58.6	3B:152216431-152216452	207	
200bp_3B_R	AGACGGGTCTTTCACTCCCG	20	59.0	3B:152216618-152216637		
250bp_4B_F	CACCGACTAGGACCTCTGAC	20	56.4	4B:437626885-437626904	250	
250bp_4B_R	GGTTCGGCGTTGTGCCGATT	20	61.3	4B:437627115-437627134		
300bp_5B_F	AGGAGTAGATGCTGCCTGCC	20	59.4	5B:63266364-63266383	300	
300bp_5B_R	GACAGCTCACACGAGACACC	20	57.9	5B:63266644-63266663		
350bp_6B_F	CAGCTGGCCGGAATTCTTCC	20	58.8	6B:150479072-150479091	350	
350bp_6B_R	GCGAGCAAGACTTGGCCAAC	20	59.5	6B:150479402-150479421		
400bp_7B_F	GCTAGGGGCGTAACCGTACT	20	58.9	7B:622973613-622973632	399	
400bp_7B_R	GGCTGCATTGGCGCAGTCGC	20	65.1	7B:622973992-622974011		
100bp_1D_F	CCTGCGCGTTACAAGTCTCG	20	58.6	1D:112473637-112473656	100	
100bp_1D_R	CCGAGACCGCCAGAAGTAAC	20	57.7	1D:112473717-112473736		
150bp_2D_F	AAGCACATGGATCGGGGCCT	20	61.9	2D:27451470-27451489	150	
150bp_2D_R	CTCCACTACGGGTCTCGCTA	20	58.0	2D:27370825-27370844		
200bp_3D_F	GCGGCGGCCTAAGATCTAAC	20	58.2	3D:103651721-103651740	200	
200bp_3D_R	GCGCCATTCTGATCTCGGCT	20	60.2	3D:103651901-103651920		
250bp_4D_F	TCTAGACTCTGACCACGCGG	20	58.4	4D:1368997-1369016	250	
250bp_4D_R	CTGCTCCAACCGCTATGACC	20	58.2	4D:1369227-1369246		
300bp_5D_F	GCCTTGGCAGAACTTCCTGC	20	59.2	5D:125349698-125349717	300	
300bp_5D_R	GACGTACTCCAGTCACCGCA	20	59.1	5D:125349978-125349997		
350bp_6D_F	TGGATGCGCATGAGCGTACG	20	60.5	6D:20108101-20108120	350	
350bp_6D_R	GAGAAGCGCACGATCGGTTG	20	58.9	6D:20108431-20108450		
400bp_7D_F	CGTACGCTAGGTCTGGATCC	20	56.8	7D:138350913-138350932	402	
400bp_7D_R	CTCCACCTGAATGGCCCGAT	20	59.7	7D:138351295-138351314		
100bp_1H_F	AAGAAGGACGGGCAGTGTCC	20	59.7	1H:30181438-30181457	103	
100bp_1H_R	GTCCAGCTGCATCTGCAGGT	20	60.3	1H:30181355-30181374		
150bp_2H_F	CGCGTCGATCGTGTCCATAC	20	58.1	2H:652788188-652788207	147	
150bp_2H_R	GCGTGCAGGCCTAATCCATC	20	58.8	2H:652788315-652788334		
200bp_3H_F	CATGTGATCTCCCACCGGCT	20	59.6	3H:246129369-246129388	200	
200bp_3H_R	GGAGCGACGTTGAGAACTGC	20	58.6	3H:246129549-246129568		
250bp_4H_F	ACAAGACCACCAACAGCAGTTCCGG	25	63.3	4H:113766350-113766374	250	
250bp_4H_R	GGGACTAGCAGCACAGGACAGG	22	63.6	4H:113766578-113766599		
300bp_5H_F	CTCTCTCTGTGGGCGCTAAC	20	57.5	5H:408498106-408498125	304	
300bp_5H_R	TAGGTGGGTCGATCCGAGGA	20	59.5	5H:408498390-408498409		
350bp_6H_F	GGTGCGACGTGATCTACACC	20	57.9	6H:56727617-56727636	352	
350bp_6H_R	GCAATGCACAGGGTGTTACTCG	22	58.7	6H:56727947-56727968		
400bp_7H_F	ATGTCCTCGAGTGCACCTGG	20	59.4	7H:186518611-186518630	399	
400bp_7H_R	TGCCTAGATACGCCTACGCG	20	59.0	7H:186518990-186519009		
350bp_6A_F2	AATGGTTGTGAGCCGCCTCG	20	60.6	6A:435829298-435829317	350	Fig. 6B
350bp_6A_R2	AATCCGGCGGCTTGGCAGAA	20	62.6	6A:435829628-435829647		Fig. 6B
400bp_7B_F2	CTCTGGACGTCAGCCTTAGC	20	57.5	7B:707147927-707147946	401	Fig. 6B
400bp_7B_R2	CGTGAGGTCCCATAGAGTGG	20	56.9	7B:707148308-707148327		Fig. 6B
Range:		20-25 nt	56.4-65.1℃		100-402 bp	
Primers designed by the refined protocol (see Materials and Methods)						

Table S4- *In silico* prediction chromosome-specific MPCR products in 16 wheat and two barley genomes

	Potential unspecific or missing PCR product		12-bp insertion			
	Plex-a; Triticum aestivum / Chinese Spring					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	1A dna:chromosome chromosome:IWGSC:1A:1:594102056:1 REF	538121084	538121183	100bp_F_1A::100bp_R_1A	99	0::0
CHR-2	2A dna:chromosome chromosome:IWGSC:2A:1:780798557:1 REF	722058206	722058355	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	3A dna:chromosome chromosome:IWGSC:3A:1:750843639:1 REF	381302864	381303063	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	4A dna:chromosome chromosome:IWGSC:4A:1:744588157:1 REF	193096666	193096915	250bp_F_4A::250bp_R_4A	249	0::0
CHR-5	5A dna:chromosome chromosome:IWGSC:5A:1:709773743:1 REF	32789504	32789803	300bp_F_5A::300bp_R_5A	299	0::0
CHR-6	6A dna:chromosome chromosome:IWGSC:6A:1:618079260:1 REF	605537994	605538343	350bp_F_6A::350bp_R_6A	349	0::0
CHR-7	7A dna:chromosome chromosome:IWGSC:7A:1:736706236:1 REF	433054330	433054729	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / Chinese Spring					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	1B dna:chromosome chromosome:IWGSC:1B:1:689851870:1 REF	102525056	102525155	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2	2B dna:chromosome chromosome:IWGSC:2B:1:801256715:1 REF	649462075	649462224	150bp_F_2B::150bp_R_2B	149	0::0
CHR-3	3B dna:chromosome chromosome:IWGSC:3B:1:830829764:1 REF	152216431	152216637	200bp_F_3B::200bp_R_3B	206	0::0
CHR-4	4B dna:chromosome chromosome:IWGSC:4B:1:673617499:1 REF	437626885	437627134	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	5B dna:chromosome chromosome:IWGSC:5B:1:713149757:1 REF	63266364	63266663	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	6B dna:chromosome chromosome:IWGSC:6B:1:720988478:1 REF	150479072	150479421	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	7B dna:chromosome chromosome:IWGSC:7B:1:750620385:1 REF	622973613	622974011	400bp_F_7B::400bp_R_7B	398	0::0
	Plex-d; Triticum aestivum / Chinese Spring					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	1D dna:chromosome chromosome:IWGSC:1D:1:495453186:1 REF	112473637	112473736	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	2D dna:chromosome chromosome:IWGSC:2D:1:651852609:1 REF	27451470	27451619	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	3D dna:chromosome chromosome:IWGSC:3D:1:615552423:1 REF	103651721	103651920	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	4D dna:chromosome chromosome:IWGSC:4D:1:509857067:1 REF	1368997	1369246	250bp_F_4D::250bp_R_4D	249	0::0
CHR-5	5D dna:chromosome chromosome:IWGSC:5D:1:566080677:1 REF	125349698	125349997	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	6D dna:chromosome chromosome:IWGSC:6D:1:473592718:1 REF	20108101	20108450	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	7D dna:chromosome chromosome:IWGSC:7D:1:638686055:1 REF	138350913	138351314	400bp_F_7D::400bp_R_7D	401	0::0
	Plex-h; Triticum aestivum / Chinese Spring					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Triticum aestivum / Fielder					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	OU015721.1 Triticum aestivum genome assembly, chromosome: 1A	547698668	547698767	100bp_F_1A::100bp_R_1A	99	0::0
CHR-2	OU015724.1 Triticum aestivum genome assembly, chromosome: 2A	726285518	726285667	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	OU015727.1 Triticum aestivum genome assembly, chromosome: 3A	390062955	390063154	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	OU015730.1 Triticum aestivum genome assembly, chromosome: 4A	192158572	192158821	250bp_F_4A::250bp_R_4A	249	0::0
CHR-5	OU015733.1 Triticum aestivum genome assembly, chromosome: 5A	36907045	36907356	300bp_F_5A::300bp_R_5A	311	0::0
CHR-6	OU015736.1 Triticum aestivum genome assembly, chromosome: 6A	610035359	610035705	350bp_F_6A::350bp_R_6A	346	0::0
CHR-7	OU015739.1 Triticum aestivum genome assembly, chromosome: 7A	447389355	447389754	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / Fielder					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	OU015722.1 Triticum aestivum genome assembly, chromosome: 1B	115205580	115205679	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2	OU015725.1 Triticum aestivum genome assembly, chromosome: 2B	653878077	653878226	150bp_F_2B::150bp_R_2B	149	0::0
CHR-3	OU015728.1 Triticum aestivum genome assembly, chromosome: 3B	776473833	776474033	200bp_R_3B::200bp_F_3B	200	0::0
CHR-4	OU015731.1 Triticum aestivum genome assembly, chromosome: 4B	451334968	451335217	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	OU015734.1 Triticum aestivum genome assembly, chromosome: 5B	68272763	68273062	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	OU015737.1 Triticum aestivum genome assembly, chromosome: 6B	152185721	152186070	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	OU015740.1 Triticum aestivum genome assembly, chromosome: 7B	625039761	625040161	400bp_F_7B::400bp_R_7B	400	0::0

	Plex-d; Triticum aestivum / Fielder					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	OU015723.1 Triticum aestivum genome assembly, chromosome: 1D	115899875	115899974	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	OU015726.1 Triticum aestivum genome assembly, chromosome: 2D	31735030	31735179	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	OU015729.1 Triticum aestivum genome assembly, chromosome: 3D	124823261	124823460	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	OU015732.1 Triticum aestivum genome assembly, chromosome: 4D	703004	703253	250bp_F_4D::250bp_R_4D	249	0::0
CHR-5	OU015735.1 Triticum aestivum genome assembly, chromosome: 5D	132997397	132997696	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	CAJRHR010000027.1 Triticum aestivum genome assembly, contig: c	144844	145193	350bp_R_6D::350bp_F_6D	349	0::0
CHR-7	OU015741.1 Triticum aestivum genome assembly, chromosome: 7D	456441797	456442196	400bp_F_7D::400bp_R_7D	399	0::0
	Plex-h; Triticum aestivum / Fielder					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Triticum aestivum / LRPB Lancer					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR862529.1 Triticum aestivum genome assembly, chromosome: 1A	538832196	538832295	100bp_F_1A::100bp_R_1A	99	0::0
CHR-2	LR862532.1 Triticum aestivum genome assembly, chromosome: 2A	717127773	717127922	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	LR862535.1 Triticum aestivum genome assembly, chromosome: 3A	386032267	386032466	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	LR862538.1 Triticum aestivum genome assembly, chromosome: 4A	192630502	192630751	250bp_F_4A::250bp_R_4A	249	0::0
CHR-5	LR862541.1 Triticum aestivum genome assembly, chromosome: 5A	36373095	36373406	300bp_F_5A::300bp_R_5A	311	0::0
CHR-6	LR862544.1 Triticum aestivum genome assembly, chromosome: 6A	597385926	597386275	350bp_F_6A::350bp_R_6A	349	0::0
CHR-7	LR862547.1 Triticum aestivum genome assembly, chromosome: 7A	432968446	432968845	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / LRPB Lancer					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR862530.1 Triticum aestivum genome assembly, chromosome: 1B	104401474	104401573	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2						
CHR-3	LR862536.1 Triticum aestivum genome assembly, chromosome: 3B	158361928	158362134	200bp_F_3B::200bp_R_3B	206	0::0
CHR-4	LR862539.1 Triticum aestivum genome assembly, chromosome: 4B	431156639	431156888	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	LR862542.1 Triticum aestivum genome assembly, chromosome: 5B	68870092	68870391	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	LR862545.1 Triticum aestivum genome assembly, chromosome: 6B	144424428	144424777	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	LR862548.1 Triticum aestivum genome assembly, chromosome: 7B	612504000	612504398	400bp_F_7B::400bp_R_7B	398	0::0
	Plex-d; Triticum aestivum / LRPB Lancer					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment lengt	mismatch (F::R
CHR-1	LR862531.1 Triticum aestivum genome assembly, chromosome: 1D	111618518	111618617	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	LR862534.1 Triticum aestivum genome assembly, chromosome: 2D	28554734	28554883	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	LR862537.1 Triticum aestivum genome assembly, chromosome: 3D	107810480	107810679	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	LR862540.1 Triticum aestivum genome assembly, chromosome: 4D	2713888	2714137	250bp_R_4D::250bp_F_4D	249	0::0
CHR-5	LR862543.1 Triticum aestivum genome assembly, chromosome: 5D	127096256	127096555	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	LR862546.1 Triticum aestivum genome assembly, chromosome: 6D	18455558	18455907	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	LR862549.1 Triticum aestivum genome assembly, chromosome: 7D	138800385	138800786	400bp_F_7D::400bp_R_7D	401	0::0
Off-target	LR862539.1 Triticum aestivum genome assembly, chromosome: 4B	1704295	1704537	250bp_R_4D::250bp_F_4D	242	1::2
	Plex-h; Triticum aestivum / LRPB Lancer					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						

	Plex-a; Triticum aestivum / CDC Stanley					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR865739.1 Triticum aestivum genome assembly, chromosome: 1A	539826757	539826856	100bp_F_1A::100bp_R_1A	99	0::0
CHR-2	LR865742.1 Triticum aestivum genome assembly, chromosome: 2A	738229692	738229841	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	LR865745.1 Triticum aestivum genome assembly, chromosome: 3A	394975678	394975877	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	LR865748.1 Triticum aestivum genome assembly, chromosome: 4A	194143828	194144077	250bp_F_4A::250bp_R_4A	249	0::0
CHR-5	LR865751.1 Triticum aestivum genome assembly, chromosome: 5A	36467940	36468251	300bp_F_5A::300bp_R_5A	311	0::0
CHR-6	LR865754.1 Triticum aestivum genome assembly, chromosome: 6A	610228205	610228554	350bp_F_6A::350bp_R_6A	349	0::0
CHR-7	LR865757.1 Triticum aestivum genome assembly, chromosome: 7A	437838977	437839376	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / CDC Stanley					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR865740.1 Triticum aestivum genome assembly, chromosome: 1B	107090560	107090659	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2	LR865743.1 Triticum aestivum genome assembly, chromosome: 2B	652816759	652816908	150bp_F_2B::150bp_R_2B	149	0::0
CHR-3	LR865746.1 Triticum aestivum genome assembly, chromosome: 3B	164453106	164453312	200bp_F_3B::200bp_R_3B	206	0::0
CHR-4	LR865749.1 Triticum aestivum genome assembly, chromosome: 4B	456778430	456778679	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	LR865752.1 Triticum aestivum genome assembly, chromosome: 5B	68190942	68191241	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	LR865755.1 Triticum aestivum genome assembly, chromosome: 6B	154861016	154861365	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	LR865758.1 Triticum aestivum genome assembly, chromosome: 7B	626207864	626208262	400bp_F_7B::400bp_R_7B	398	0::0
	Plex-d; Triticum aestivum / CDC Stanley					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR865741.1 Triticum aestivum genome assembly, chromosome: 1D	114265840	114265939	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	LR865744.1 Triticum aestivum genome assembly, chromosome: 2D	28764218	28764367	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	LR865747.1 Triticum aestivum genome assembly, chromosome: 3D	114491893	114492092	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	LR865750.1 Triticum aestivum genome assembly, chromosome: 4D	1022984	1023233	250bp_F_4D::250bp_R_4D	249	0::0
CHR-5	LR865753.1 Triticum aestivum genome assembly, chromosome: 5D	128937771	128938070	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	LR865756.1 Triticum aestivum genome assembly, chromosome: 6D	21178328	21178677	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	LR865759.1 Triticum aestivum genome assembly, chromosome: 7D	140981738	140982139	400bp_F_7D::400bp_R_7D	401	0::0
	Plex-h; Triticum aestivum / CDC Stanley					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Triticum aestivum / Paragon					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYP010083961|CADDYP010083961.1 Triticum aestivum	41633	41732	100bp_R_1A::100bp_F_1A	99	0::0
CHR-2	ENA|CADDYP010106770|CADDYP010106770.1 Triticum aestivum	16826	16975	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	ENA|CADDYP010017895|CADDYP010017895.1 Triticum aestivum	53090	53289	200bp_R_3A::200bp_F_3A	199	0::0
CHR-4	ENA|CADDYP010042964|CADDYP010042964.1 Triticum aestivum	17442	17691	250bp_R_4A::250bp_F_4A	249	0::0
CHR-5	ENA|CADDYP010289979|CADDYP010289979.1 Triticum aestivum	3155	3466	300bp_R_5A::300bp_F_5A	311	0::0
CHR-6	ENA|CADDYP010021890|CADDYP010021890.1 Triticum aestivum	113930	114279	350bp_R_6A::350bp_F_6A	349	0::0
CHR-7	ENA|CADDYP010026057|CADDYP010026057.1 Triticum aestivum	53026	53425	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / Paragon					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYP010001383|CADDYP010001383.1 Triticum aestivum	254146	254245	100bp_R_1B::100bp_F_1B	99	0::0
CHR-2	ENA|CADDYP010036909|CADDYP010036909.1 Triticum aestivum	55346	55495	150bp_F_2B::150bp_R_2B	149	1::0
CHR-3	ENA|CADDYP010031776|CADDYP010031776.1 Triticum aestivum	16744	16950	200bp_F_3B::200bp_R_3B	206	0::0
CHR-4	ENA|CADDYP010000591|CADDYP010000591.1 Triticum aestivum	205779	206028	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	ENA|CADDYP010042620|CADDYP010042620.1 Triticum aestivum	37005	37304	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	ENA|CADDYP010002282|CADDYP010002282.1 Triticum aestivum	133858	134207	350bp_R_6B::350bp_F_6B	349	0::0
CHR-7	ENA|CADDYP010051281|CADDYP010051281.1 Triticum aestivum	70087	70485	400bp_R_7B::400bp_F_7B	398	0::0

	Plex-d; Triticum aestivum / Paragon					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYP010062483|CADDYP010062483.1 Triticum aestivum	50558	50657	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	ENA|CADDYP010167131|CADDYP010167131.1 Triticum aestivum	10832	10981	150bp_R_2D::150bp_F_2D	149	0::0
CHR-3	ENA|CADDYP010009997|CADDYP010009997.1 Triticum aestivum	94727	94926	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	ENA|CADDYP010035697|CADDYP010035697.1 Triticum aestivum	51119	51368	250bp_R_4D::250bp_F_4D	249	0::0
CHR-5	ENA|CADDYP010067139|CADDYP010067139.1 Triticum aestivum	42108	42407	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	ENA|CADDYP010117606|CADDYP010117606.1 Triticum aestivum	2640	2989	350bp_R_6D::350bp_F_6D	349	0::0
CHR-7	ENA|CADDYP010127897|CADDYP010127897.1 Triticum aestivum	1667	2068	400bp_R_7D::400bp_F_7D	401	0::0
Off-target	ENA|CADDYP010023539|CADDYP010023539.1 Triticum aestivum	96624	96866	250bp_R_4D::250bp_F_4D	242	1::2
	Plex-h; Triticum aestivum / Paragon					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Triticum aestivum / SY Mattis					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR865760.1 Triticum aestivum genome assembly, chromosome: 1A	543105337	543105436	100bp_F_1A::100bp_R_1A	99	0::0
CHR-2	LR865763.1 Triticum aestivum genome assembly, chromosome: 2A	725012284	725012434	150bp_F_2A::150bp_R_2A	150	0::0
CHR-3	LR865766.1 Triticum aestivum genome assembly, chromosome: 3A	388025053	388025252	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	LR865769.1 Triticum aestivum genome assembly, chromosome: 4A	191743987	191744236	250bp_F_4A::250bp_R_4A	249	0::0
CHR-5	LR865772.1 Triticum aestivum genome assembly, chromosome: 5A	35067255	35067554	300bp_F_5A::300bp_R_5A	299	0::0
CHR-6	LR865775.1 Triticum aestivum genome assembly, chromosome: 6A	599380377	599380726	350bp_F_6A::350bp_R_6A	349	0::0
CHR-7	LR865778.1 Triticum aestivum genome assembly, chromosome: 7A	433782472	433782871	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / SY Mattis					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR865761.1 Triticum aestivum genome assembly, chromosome: 1B	101952457	101952556	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2	LR865764.1 Triticum aestivum genome assembly, chromosome: 2B	649008971	649009120	150bp_F_2B::150bp_R_2B	149	1::0
CHR-3	LR865767.1 Triticum aestivum genome assembly, chromosome: 3B	157014831	157015037	200bp_F_3B::200bp_R_3B	206	0::0
CHR-4	LR865770.1 Triticum aestivum genome assembly, chromosome: 4B	431144235	431144484	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	LR865773.1 Triticum aestivum genome assembly, chromosome: 5B	61617435	61617734	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	LR865776.1 Triticum aestivum genome assembly, chromosome: 6B	150224398	150224747	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	LR865779.1 Triticum aestivum genome assembly, chromosome: 7B	841487024	841487422	400bp_F_7B::400bp_R_7B	398	0::0
	Plex-d; Triticum aestivum / SY Mattis					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR865762.1 Triticum aestivum genome assembly, chromosome: 1D	110559101	110559200	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	LR865765.1 Triticum aestivum genome assembly, chromosome: 2D	28263597	28263746	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	LR865768.1 Triticum aestivum genome assembly, chromosome: 3D	102083833	102084032	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	LR865771.1 Triticum aestivum genome assembly, chromosome: 4D	1245162	1245411	250bp_R_4D::250bp_F_4D	249	0::0
CHR-5	LR865774.1 Triticum aestivum genome assembly, chromosome: 5D	126344263	126344562	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	LR865777.1 Triticum aestivum genome assembly, chromosome: 6D	19784209	19784558	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	LR865780.1 Triticum aestivum genome assembly, chromosome: 7D	137057836	137058237	400bp_F_7D::400bp_R_7D	401	0::0
Off-target	LR865770.1 Triticum aestivum genome assembly, chromosome: 4B	10701458	10701700	250bp_F_4D::250bp_R_4D	242	2::1
	Plex-h; Triticum aestivum / SY Mattis					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						

	Plex-a; Triticum aestivum / Julius					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR865500.1 Triticum aestivum genome assembly, chromosome: 1A	534519185	534519284	100bp_F_1A::100bp_R_1A	99	0::0
CHR-2	LR865503.1 Triticum aestivum genome assembly, chromosome: 2A	730543185	730543334	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	LR865506.1 Triticum aestivum genome assembly, chromosome: 3A	394066637	394066836	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	LR865509.1 Triticum aestivum genome assembly, chromosome: 4A	193540226	193540475	250bp_F_4A::250bp_R_4A	249	0::0
CHR-5	LR865512.1 Triticum aestivum genome assembly, chromosome: 5A	36860098	36860397	300bp_F_5A::300bp_R_5A	299	0::0
CHR-6	LR865515.1 Triticum aestivum genome assembly, chromosome: 6A	606608209	606608558	350bp_F_6A::350bp_R_6A	349	1::0
CHR-7	LR865518.1 Triticum aestivum genome assembly, chromosome: 7A	438366876	438367275	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / Julius					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR865501.1 Triticum aestivum genome assembly, chromosome: 1B	105965104	105965203	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2	LR865504.1 Triticum aestivum genome assembly, chromosome: 2B	651328574	651328723	150bp_F_2B::150bp_R_2B	149	1::0
CHR-3	LR865507.1 Triticum aestivum genome assembly, chromosome: 3B	162137251	162137457	200bp_F_3B::200bp_R_3B	206	0::0
CHR-4	LR865510.1 Triticum aestivum genome assembly, chromosome: 4B	431318671	431318920	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	LR865513.1 Triticum aestivum genome assembly, chromosome: 5B	73894155	73894454	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	LR865516.1 Triticum aestivum genome assembly, chromosome: 6B	154603099	154603448	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	LR865519.1 Triticum aestivum genome assembly, chromosome: 7B	622339246	622339644	400bp_F_7B::400bp_R_7B	398	0::0
	Plex-d; Triticum aestivum / Julius					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR865502.1 Triticum aestivum genome assembly, chromosome: 1D	112327222	112327321	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	LR865505.1 Triticum aestivum genome assembly, chromosome: 2D	32743620	32743769	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	LR865508.1 Triticum aestivum genome assembly, chromosome: 3D	108531180	108531379	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	LR865511.1 Triticum aestivum genome assembly, chromosome: 4D	1227131	1227380	250bp_R_4D::250bp_F_4D	249	0::0
CHR-5	LR865514.1 Triticum aestivum genome assembly, chromosome: 5D	128642058	128642357	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	LR865517.1 Triticum aestivum genome assembly, chromosome: 6D	20026996	20027345	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	LR865520.1 Triticum aestivum genome assembly, chromosome: 7D	140937120	140937521	400bp_F_7D::400bp_R_7D	401	0::0
Off-target	LR865510.1 Triticum aestivum genome assembly, chromosome: 4B	12753090	12753332	250bp_F_4D::250bp_R_4D	242	2::1
	Plex-h; Triticum aestivum / Julius					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Triticum aestivum / Cadenza					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYN010063059|CADDYN010063059.1 Triticum aestivur	49392	49491	100bp_R_1A::100bp_F_1A	99	0::0
CHR-2	ENA|CADDYN010077897|CADDYN010077897.1 Triticum aestivur	23639	23788	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	ENA|CADDYN010019120|CADDYN010019120.1 Triticum aestivur	80991	81190	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	ENA|CADDYN010038504|CADDYN010038504.1 Triticum aestivur	40052	40301	250bp_R_4A::250bp_F_4A	249	0::0
CHR-5	ENA|CADDYN010150084|CADDYN010150084.1 Triticum aestivur	15116	15427	300bp_R_5A::300bp_F_5A	311	0::0
CHR-6	ENA|CADDYN010029415|CADDYN010029415.1 Triticum aestivur	85366	85715	350bp_R_6A::350bp_F_6A	349	0::0
CHR-7	ENA|CADDYN010000620|CADDYN010000620.1 Triticum aestivur	207710	208109	400bp_R_7A::400bp_F_7A	399	0::0
	Plex-b; Triticum aestivum / Cadenza					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYN010005447|CADDYN010005447.1 Triticum aestivur	48591	48690	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2	ENA|CADDYN010036949|CADDYN010036949.1 Triticum aestivur	55728	55877	150bp_F_2B::150bp_R_2B	149	1::0
CHR-3	ENA|CADDYN010038573|CADDYN010038573.1 Triticum aestivur	14788	14994	200bp_F_3B::200bp_R_3B	206	0::0
CHR-4	ENA|CADDYN010001282|CADDYN010001282.1 Triticum aestivur	148378	148627	250bp_R_4B::250bp_F_4B	249	0::0
CHR-5	ENA|CADDYN010020270|CADDYN010020270.1 Triticum aestivur	44705	45004	300bp_R_5B::300bp_F_5B	299	0::0
CHR-6	ENA|CADDYN010025736|CADDYN010025736.1 Triticum aestivur	101540	101889	350bp_R_6B::350bp_F_6B	349	0::0
CHR-7	ENA|CADDYN010057973|CADDYN010057973.1 Triticum aestivur	5088	5486	400bp_F_7B::400bp_R_7B	398	0::0

	Plex-d; Triticum aestivum / Cadenza					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYN010048251|CADDYN010048251.1 Triticum aestivur	55361	55460	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	ENA|CADDYN010125947|CADDYN010125947.1 Triticum aestivur	14568	14717	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	ENA|CADDYN010072714|CADDYN010072714.1 Triticum aestivur	54668	54867	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	ENA|CADDYN010034326|CADDYN010034326.1 Triticum aestivur	49614	49863	250bp_F_4D::250bp_R_4D	249	0::0
CHR-5	ENA|CADDYN010082330|CADDYN010082330.1 Triticum aestivur	29851	30150	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	ENA|CADDYN010034395|CADDYN010034395.1 Triticum aestivur	54173	54522	350bp_R_6D::350bp_F_6D	349	0::0
CHR-7	ENA|CADDYN010025773|CADDYN010025773.1 Triticum aestivur	33086	33487	400bp_R_7D::400bp_F_7D	401	0::0
	Plex-h; Triticum aestivum / Cadenza					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Triticum aestivum / Weebill 1					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYO010070251|CADDYO010070251.1 Triticum aestivur	11983	12082	100bp_R_1A::100bp_F_1A	99	0::0
CHR-2	ENA|CADDYO010029567|CADDYO010029567.1 Triticum aestivur	8096	8245	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	ENA|CADDYO010014208|CADDYO010014208.1 Triticum aestivur	82543	82742	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	ENA|CADDYO010141296|CADDYO010141296.1 Triticum aestivur	6107	6356	250bp_F_4A::250bp_R_4A	249	0::0
CHR-5	ENA|CADDYO010044193|CADDYO010044193.1 Triticum aestivur	80259	80570	300bp_R_5A::300bp_F_5A	311	0::0
CHR-6	ENA|CADDYO010038836|CADDYO010038836.1 Triticum aestivur	83950	84299	350bp_R_6A::350bp_F_6A	349	0::0
CHR-7	ENA|CADDYO010003603|CADDYO010003603.1 Triticum aestivur	25721	26120	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / Weebill 1					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYO010001566|CADDYO010001566.1 Triticum aestivur	49049	49148	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2	ENA|CADDYO010010122|CADDYO010010122.1 Triticum aestivur	120508	120657	150bp_F_2B::150bp_R_2B	149	0::0
CHR-3	ENA|CADDYO010021368|CADDYO010021368.1 Triticum aestivur	110019	110225	200bp_R_3B::200bp_F_3B	206	0::0
CHR-4	ENA|CADDYO010003213|CADDYO010003213.1 Triticum aestivur	40563	40812	250bp_R_4B::250bp_F_4B	249	0::0
CHR-5	ENA|CADDYO010003694|CADDYO010003694.1 Triticum aestivur	180019	180318	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	ENA|CADDYO010009048|CADDYO010009048.1 Triticum aestivur	105340	105689	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	ENA|CADDYO010089804|CADDYO010089804.1 Triticum aestivur	5088	5486	400bp_F_7B::400bp_R_7B	398	0::0
	Plex-d; Triticum aestivum / Weebill 1					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYO010036676|CADDYO010036676.1 Triticum aestivur	79082	79181	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	ENA|CADDYO010063489|CADDYO010063489.1 Triticum aestivur	45918	46067	150bp_R_2D::150bp_F_2D	149	0::0
CHR-3	ENA|CADDYO010032275|CADDYO010032275.1 Triticum aestivur	50514	50713	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	ENA|CADDYO010036889|CADDYO010036889.1 Triticum aestivur	43511	43760	250bp_F_4D::250bp_R_4D	249	0::0
CHR-5	ENA|CADDYO010102848|CADDYO010102848.1 Triticum aestivur	23551	23850	300bp_R_5D::300bp_F_5D	299	0::0
CHR-6	ENA|CADDYO010042841|CADDYO010042841.1 Triticum aestivur	23746	24095	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	ENA|CADDYO010036599|CADDYO010036599.1 Triticum aestivur	56784	57185	400bp_F_7D::400bp_R_7D	401	0::0
	Plex-h; Triticum aestivum / Weebill 1					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						

	Plex-a; Triticum aestivum / Claire					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYM010040171|CADDYM010040171.1 Triticum aestivu	46465	46564	100bp_F_1A::100bp_R_1A	99	0::0
CHR-2	ENA|CADDYM010084815|CADDYM010084815.1 Triticum aestivu	18378	18527	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	ENA|CADDYM010018664|CADDYM010018664.1 Triticum aestivu	51805	52004	200bp_R_3A::200bp_F_3A	199	0::0
CHR-4	ENA|CADDYM010035066|CADDYM010035066.1 Triticum aestivu	53059	53308	250bp_F_4A::250bp_R_4A	249	0::0
CHR-5	ENA|CADDYM010006039|CADDYM010006039.1 Triticum aestivu	90263	90562	300bp_F_5A::300bp_R_5A	299	0::0
CHR-6	ENA|CADDYM010059858|CADDYM010059858.1 Triticum aestivu	7228	7574	350bp_F_6A::350bp_R_6A	346	0::0
CHR-7	ENA|CADDYM010005314|CADDYM010005314.1 Triticum aestivu	58835	59234	400bp_R_7A::400bp_F_7A	399	0::0
	Plex-b; Triticum aestivum / Claire					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYM010029048|CADDYM010029048.1 Triticum aestivu	56692	56791	100bp_R_1B::100bp_F_1B	99	0::0
CHR-2	ENA|CADDYM010036435|CADDYM010036435.1 Triticum aestivu	56011	56160	150bp_F_2B::150bp_R_2B	149	1::0
CHR-3	ENA|CADDYM010033404|CADDYM010033404.1 Triticum aestivu	13934	14140	200bp_F_3B::200bp_R_3B	206	0::0
CHR-4	ENA|CADDYM010001354|CADDYM010001354.1 Triticum aestivu	59173	59422	250bp_R_4B::250bp_F_4B	249	0::0
CHR-5	ENA|CADDYM010003687|CADDYM010003687.1 Triticum aestivu	191341	191640	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	ENA|CADDYM010061065|CADDYM010061065.1 Triticum aestivu	17762	18111	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	ENA|CADDYM010015635|CADDYM010015635.1 Triticum aestivu	51322	51720	400bp_F_7B::400bp_R_7B	398	0::0
	Plex-d; Triticum aestivum / Claire					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYM010037974|CADDYM010037974.1 Triticum aestivu	78604	78703	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	ENA|CADDYM010075457|CADDYM010075457.1 Triticum aestivu	16904	17053	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	ENA|CADDYM010002641|CADDYM010002641.1 Triticum aestivu	51083	51282	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	ENA|CADDYM010002124|CADDYM010002124.1 Triticum aestivu	47256	47505	250bp_R_4D::250bp_F_4D	249	0::0
CHR-5	ENA|CADDYM010078956|CADDYM010078956.1 Triticum aestivu	29256	29555	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	ENA|CADDYM010089688|CADDYM010089688.1 Triticum aestivu	41771	42120	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	ENA|CADDYM010032981|CADDYM010032981.1 Triticum aestivu	90067	90468	400bp_F_7D::400bp_R_7D	401	0::0
	Plex-h; Triticum aestivum / Claire					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Triticum aestivum / Robigus					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYY010038971|CADDYY010038971.1 Triticum aestivur	38912	39011	100bp_R_1A::100bp_F_1A	99	0::0
CHR-2	ENA|CADDYY010028567|CADDYY010028567.1 Triticum aestivur	17015	17164	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	ENA|CADDYY010021661|CADDYY010021661.1 Triticum aestivur	51802	52001	200bp_R_3A::200bp_F_3A	199	0::0
CHR-4	ENA|CADDYY010033845|CADDYY010033845.1 Triticum aestivur	59969	60218	250bp_F_4A::250bp_R_4A	249	0::0
CHR-5	ENA|CADDYY010006606|CADDYY010006606.1 Triticum aestivur	140578	140877	300bp_F_5A::300bp_R_5A	299	0::0
CHR-6	ENA|CADDYY010062909|CADDYY010062909.1 Triticum aestivur	62044	62390	350bp_R_6A::350bp_F_6A	346	0::0
CHR-7	ENA|CADDYY010000967|CADDYY010000967.1 Triticum aestivur	146813	147212	400bp_F_7A::400bp_R_7A	399	0::0
Off-target	ENA|CADDYY010099957|CADDYY010099957.1 Triticum aestivur	36526	36872	350bp_R_6A::350bp_F_6A	346	0::0
	Plex-b; Triticum aestivum / Robigus					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYY010002001|CADDYY010002001.1 Triticum aestivur	235956	236055	100bp_R_1B::100bp_F_1B	99	0::0
CHR-2	ENA|CADDYY010030123|CADDYY010030123.1 Triticum aestivur	104544	104693	150bp_F_2B::150bp_R_2B	149	1::0
CHR-3	ENA|CADDYY010140686|CADDYY010140686.1 Triticum aestivur	18077	18283	200bp_F_3B::200bp_R_3B	206	0::0
CHR-4	ENA|CADDYY010001007|CADDYY010001007.1 Triticum aestivur	229924	230173	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	ENA|CADDYY010010569|CADDYY010010569.1 Triticum aestivur	48632	48931	300bp_R_5B::300bp_F_5B	299	0::0
CHR-6	ENA|CADDYY010003236|CADDYY010003236.1 Triticum aestivur	26674	27023	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	ENA|CADDYY010050180|CADDYY010050180.1 Triticum aestivur	73824	74222	400bp_R_7B::400bp_F_7B	398	0::0

	Plex-d; Triticum aestivum / Robigus					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	ENA|CADDYY010063480|CADDYY010063480.1 Triticum aestivur	52922	53021	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	ENA|CADDYY010039670|CADDYY010039670.1 Triticum aestivur	58141	58290	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	ENA|CADDYY010024324|CADDYY010024324.1 Triticum aestivur	65442	65641	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	ENA|CADDYY010035204|CADDYY010035204.1 Triticum aestivur	53413	53662	250bp_R_4D::250bp_F_4D	249	0::0
CHR-5	ENA|CADDYY010073358|CADDYY010073358.1 Triticum aestivur	30995	31294	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	ENA|CADDYY010035649|CADDYY010035649.1 Triticum aestivur	36905	37254	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	ENA|CADDYY010037362|CADDYY010037362.1 Triticum aestivur	56351	56752	400bp_F_7D::400bp_R_7D	401	0::0
	Plex-h; Triticum aestivum / Robigus					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Triticum aestivum / Jagger					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR862508.1 Triticum aestivum genome assembly, chromosome: 1A	539548936	539549035	100bp_F_1A::100bp_R_1A	99	0::0
CHR-2	LR862511.1 Triticum aestivum genome assembly, chromosome: 2A	738825024	738825173	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	LR862514.1 Triticum aestivum genome assembly, chromosome: 3A	391562383	391562582	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	LR862517.1 Triticum aestivum genome assembly, chromosome: 4A	193114146	193114394	250bp_F_4A::250bp_R_4A	248	0::0
CHR-5	LR862520.1 Triticum aestivum genome assembly, chromosome: 5A	35277046	35277357	300bp_F_5A::300bp_R_5A	311	0::0
CHR-6	LR862523.1 Triticum aestivum genome assembly, chromosome: 6A	610853057	610853406	350bp_F_6A::350bp_R_6A	349	0::0
CHR-7	LR862526.1 Triticum aestivum genome assembly, chromosome: 7A	437883279	437883678	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / Jagger					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR862509.1 Triticum aestivum genome assembly, chromosome: 1B	104392943	104393042	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2	LR862512.1 Triticum aestivum genome assembly, chromosome: 2B	649322006	649322155	150bp_F_2B::150bp_R_2B	149	1::0
CHR-3	LR862515.1 Triticum aestivum genome assembly, chromosome: 3B	164350197	164350403	200bp_F_3B::200bp_R_3B	206	0::0
CHR-4	LR862518.1 Triticum aestivum genome assembly, chromosome: 4B	437321896	437322145	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	LR862521.1 Triticum aestivum genome assembly, chromosome: 5B	65653271	65653570	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	LR862524.1 Triticum aestivum genome assembly, chromosome: 6B	154649463	154649812	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	LR862527.1 Triticum aestivum genome assembly, chromosome: 7B	623197595	623197993	400bp_F_7B::400bp_R_7B	398	0::0
	Plex-d; Triticum aestivum / Jagger					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR862510.1 Triticum aestivum genome assembly, chromosome: 1D	110330910	110331009	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	LR862513.1 Triticum aestivum genome assembly, chromosome: 2D	28741234	28741383	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	LR862516.1 Triticum aestivum genome assembly, chromosome: 3D	103861240	103861439	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	LR862519.1 Triticum aestivum genome assembly, chromosome: 4D	9340050	9340299	250bp_F_4D::250bp_R_4D	249	0::0
CHR-5	LR862522.1 Triticum aestivum genome assembly, chromosome: 5D	128122721	128123020	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	LR862525.1 Triticum aestivum genome assembly, chromosome: 6D	19548554	19548903	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	LR862528.1 Triticum aestivum genome assembly, chromosome: 7D	138333109	138333510	400bp_F_7D::400bp_R_7D	401	0::0
	Plex-h; Triticum aestivum / Jagger					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						

	Plex-a; Triticum aestivum / ArinaLrFor					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR862550.1 Triticum aestivum genome assembly, chromosome: 1A	545204040	545204139	100bp_F_1A::100bp_R_1A	99	0::0
CHR-2	LR862553.1 Triticum aestivum genome assembly, chromosome: 2A	723432920	723433069	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	LR862556.1 Triticum aestivum genome assembly, chromosome: 3A	389006315	389006514	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	LR862559.1 Triticum aestivum genome assembly, chromosome: 4A	192559555	192559804	250bp_F_4A::250bp_R_4A	249	0::0
CHR-5	LR862562.1 Triticum aestivum genome assembly, chromosome: 5A	35133711	35134010	300bp_F_5A::300bp_R_5A	299	0::0
CHR-6	LR862565.1 Triticum aestivum genome assembly, chromosome: 6A	604986903	604987252	350bp_F_6A::350bp_R_6A	349	0::0
CHR-7	LR862568.1 Triticum aestivum genome assembly, chromosome: 7A	447708348	447708747	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / ArinaLrFor					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR862551.1 Triticum aestivum genome assembly, chromosome: 1B	99852536	99852635	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2	LR862554.1 Triticum aestivum genome assembly, chromosome: 2B	653716130	653716279	150bp_F_2B::150bp_R_2B	149	1::0
CHR-3	LR862557.1 Triticum aestivum genome assembly, chromosome: 3B	167137567	167137773	200bp_F_3B::200bp_R_3B	206	0::0
CHR-4	LR862560.1 Triticum aestivum genome assembly, chromosome: 4B	439122127	439122376	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	LR862563.1 Triticum aestivum genome assembly, chromosome: 5B	69074640	69074939	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	LR862566.1 Triticum aestivum genome assembly, chromosome: 6B	150508697	150509046	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	LR862569.1 Triticum aestivum genome assembly, chromosome: 7B	131439643	131440041	400bp_R_7B::400bp_F_7B	398	0::0
	Plex-d; Triticum aestivum / ArinaLrFor					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR862552.1 Triticum aestivum genome assembly, chromosome: 1D	111225111	111225210	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	LR862555.1 Triticum aestivum genome assembly, chromosome: 2D	29253040	29253189	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	LR862558.1 Triticum aestivum genome assembly, chromosome: 3D	109443384	109443583	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	LR862561.1 Triticum aestivum genome assembly, chromosome: 4D	1678590	1678839	250bp_R_4D::250bp_F_4D	249	0::0
CHR-5	LR862564.1 Triticum aestivum genome assembly, chromosome: 5D	128166941	128167240	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	LR862567.1 Triticum aestivum genome assembly, chromosome: 6D	21145867	21146216	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	LR862570.1 Triticum aestivum genome assembly, chromosome: 7D	140085589	140085990	400bp_F_7D::400bp_R_7D	401	0::0
	Plex-h; Triticum aestivum / ArinaLrFor					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Triticum aestivum / CDC Landmark					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR877309.1 Triticum aestivum genome assembly, chromosome: 1A	537464428	537464527	100bp_F_1A::100bp_R_1A	99	0::0
CHR-2	LR877312.1 Triticum aestivum genome assembly, chromosome: 2A	729475875	729476025	150bp_F_2A::150bp_R_2A	150	0::0
CHR-3	LR877315.1 Triticum aestivum genome assembly, chromosome: 3A	390293097	390293296	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	LR877318.1 Triticum aestivum genome assembly, chromosome: 4A	193553949	193554198	250bp_F_4A::250bp_R_4A	249	0::0
CHR-5	LR877321.1 Triticum aestivum genome assembly, chromosome: 5A	36811695	36812006	300bp_F_5A::300bp_R_5A	311	0::0
CHR-6	LR877324.1 Triticum aestivum genome assembly, chromosome: 6A	604563457	604563806	350bp_F_6A::350bp_R_6A	349	0::0
CHR-7	LR877327.1 Triticum aestivum genome assembly, chromosome: 7A	434597096	434597495	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / CDC Landmark					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR877310.1 Triticum aestivum genome assembly, chromosome: 1B	102786561	102786660	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2	LR877313.1 Triticum aestivum genome assembly, chromosome: 2B	647149703	647149852	150bp_F_2B::150bp_R_2B	149	0::0
CHR-3	LR877316.1 Triticum aestivum genome assembly, chromosome: 3B	160270042	160270248	200bp_F_3B::200bp_R_3B	206	0::0
CHR-4	LR877319.1 Triticum aestivum genome assembly, chromosome: 4B	450037024	450037273	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	LR877322.1 Triticum aestivum genome assembly, chromosome: 5B	64423385	64423684	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	LR877325.1 Triticum aestivum genome assembly, chromosome: 6B	153907336	153907685	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	LR877328.1 Triticum aestivum genome assembly, chromosome: 7B	629202294	629202692	400bp_F_7B::400bp_R_7B	398	0::0

	Plex-d; Triticum aestivum / CDC Landmark					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR877311.1 Triticum aestivum genome assembly, chromosome: 1D	114951222	114951321	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	LR877314.1 Triticum aestivum genome assembly, chromosome: 2D	29247581	29247730	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	LR877317.1 Triticum aestivum genome assembly, chromosome: 3D	107268264	107268463	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	LR877320.1 Triticum aestivum genome assembly, chromosome: 4D	2553666	2553915	250bp_R_4D::250bp_F_4D	249	0::0
CHR-5	LR877323.1 Triticum aestivum genome assembly, chromosome: 5D	128182349	128182648	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	LR877326.1 Triticum aestivum genome assembly, chromosome: 6D	20103212	20103561	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	LR877329.1 Triticum aestivum genome assembly, chromosome: 7D	140247744	140248145	400bp_F_7D::400bp_R_7D	401	0::0
	Plex-h; Triticum aestivum / CDC Landmark					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Triticum aestivum / Mace					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR865458.1 Triticum aestivum genome assembly, chromosome: 1A	534817559	534817658	100bp_F_1A::100bp_R_1A	99	0::0
CHR-2	LR865461.1 Triticum aestivum genome assembly, chromosome: 2A	722575687	722575836	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	LR865464.1 Triticum aestivum genome assembly, chromosome: 3A	380666699	380666898	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	LR865467.1 Triticum aestivum genome assembly, chromosome: 4A	191410531	191410780	250bp_F_4A::250bp_R_4A	249	0::0
CHR-5	LR865470.1 Triticum aestivum genome assembly, chromosome: 5A	36441755	36442066	300bp_F_5A::300bp_R_5A	311	0::0
CHR-6	LR865473.1 Triticum aestivum genome assembly, chromosome: 6A	597197454	597197803	350bp_F_6A::350bp_R_6A	349	0::0
CHR-7	LR865476.1 Triticum aestivum genome assembly, chromosome: 7A	432629253	432629652	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / Mace					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR865459.1 Triticum aestivum genome assembly, chromosome: 1B	107085209	107085308	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2	LR865462.1 Triticum aestivum genome assembly, chromosome: 2B	642231548	642231697	150bp_F_2B::150bp_R_2B	149	0::0
CHR-3	LR865465.1 Triticum aestivum genome assembly, chromosome: 3B	165786593	165786799	200bp_R_3B::200bp_F_3B	206	0::0
CHR-4	LR865468.1 Triticum aestivum genome assembly, chromosome: 4B	436332972	436333221	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	LR865471.1 Triticum aestivum genome assembly, chromosome: 5B	68065307	68065606	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	LR865474.1 Triticum aestivum genome assembly, chromosome: 6B	149253658	149254007	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	LR865477.1 Triticum aestivum genome assembly, chromosome: 7B	614793163	614793561	400bp_F_7B::400bp_R_7B	398	0::0
	Plex-d; Triticum aestivum / Mace					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR865460.1 Triticum aestivum genome assembly, chromosome: 1D	111292905	111293004	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	LR865463.1 Triticum aestivum genome assembly, chromosome: 2D	28238206	28238355	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	LR865466.1 Triticum aestivum genome assembly, chromosome: 3D	103585708	103585907	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	LR865469.1 Triticum aestivum genome assembly, chromosome: 4D	1060269	1060518	250bp_R_4D::250bp_F_4D	249	0::0
CHR-5	LR865472.1 Triticum aestivum genome assembly, chromosome: 5D	126432545	126432844	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	LR865475.1 Triticum aestivum genome assembly, chromosome: 6D	20082640	20082989	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	LR865478.1 Triticum aestivum genome assembly, chromosome: 7D	140308384	140308785	400bp_F_7D::400bp_R_7D	401	0::0
	Plex-h; Triticum aestivum / Mace					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						

	Plex-a; Triticum aestivum / Norin 61					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR878435.1 Triticum aestivum genome assembly, chromosome: 1A	538075659	538075758	100bp_F_1A::100bp_R_1A	99	0::0
CHR-2	LR878438.1 Triticum aestivum genome assembly, chromosome: 2A	720879480	720879629	150bp_F_2A::150bp_R_2A	149	0::0
CHR-3	LR878441.1 Triticum aestivum genome assembly, chromosome: 3A	394562344	394562543	200bp_F_3A::200bp_R_3A	199	0::0
CHR-4	LR878444.1 Triticum aestivum genome assembly, chromosome: 4A	191624561	191624809	250bp_F_4A::250bp_R_4A	248	0::0
CHR-5	LR878447.1 Triticum aestivum genome assembly, chromosome: 5A	34782171	34782470	300bp_F_5A::300bp_R_5A	299	0::0
CHR-6	LR878450.1 Triticum aestivum genome assembly, chromosome: 6A	606095532	606095881	350bp_F_6A::350bp_R_6A	349	0::0
CHR-7	LR878453.1 Triticum aestivum genome assembly, chromosome: 7A	431784958	431785357	400bp_F_7A::400bp_R_7A	399	0::0
	Plex-b; Triticum aestivum / Norin 61					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR878436.1 Triticum aestivum genome assembly, chromosome: 1B	105237590	105237689	100bp_F_1B::100bp_R_1B	99	0::0
CHR-2	LR878439.1 Triticum aestivum genome assembly, chromosome: 2B	652663841	652663990	150bp_F_2B::150bp_R_2B	149	0::0
CHR-3	LR878442.1 Triticum aestivum genome assembly, chromosome: 3B	159029929	159030135	200bp_R_3B::200bp_F_3B	206	0::0
CHR-4	LR878445.1 Triticum aestivum genome assembly, chromosome: 4B	435111051	435111300	250bp_F_4B::250bp_R_4B	249	0::0
CHR-5	LR878448.1 Triticum aestivum genome assembly, chromosome: 5B	68690360	68690659	300bp_F_5B::300bp_R_5B	299	0::0
CHR-6	LR878451.1 Triticum aestivum genome assembly, chromosome: 6B	149018958	149019307	350bp_F_6B::350bp_R_6B	349	0::0
CHR-7	LR878454.1 Triticum aestivum genome assembly, chromosome: 7B	625822371	625822769	400bp_F_7B::400bp_R_7B	398	0::0
	Plex-d; Triticum aestivum / Norin 61					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	LR878437.1 Triticum aestivum genome assembly, chromosome: 1D	112522933	112523032	100bp_F_1D::100bp_R_1D	99	0::0
CHR-2	LR878440.1 Triticum aestivum genome assembly, chromosome: 2D	28120436	28120585	150bp_F_2D::150bp_R_2D	149	0::0
CHR-3	LR878443.1 Triticum aestivum genome assembly, chromosome: 3D	106622791	106622990	200bp_F_3D::200bp_R_3D	199	0::0
CHR-4	LR878446.1 Triticum aestivum genome assembly, chromosome: 4D	1009837	1010086	250bp_F_4D::250bp_R_4D	249	0::0
CHR-5	LR878449.1 Triticum aestivum genome assembly, chromosome: 5D	124847847	124848146	300bp_F_5D::300bp_R_5D	299	0::0
CHR-6	LR878452.1 Triticum aestivum genome assembly, chromosome: 6D	20063320	20063669	350bp_F_6D::350bp_R_6D	349	0::0
CHR-7	LR878455.1 Triticum aestivum genome assembly, chromosome: 7D	137936441	137936842	400bp_F_7D::400bp_R_7D	401	0::0
	Plex-h; Triticum aestivum / Norin 61					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Hordeum vulgare / Golden Promise (Ref v1)					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-b; Hordeum vulgare / Golden Promise (Ref v1)					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						

	Plex-d; Hordeum vulgare / Golden Promise (Ref v1)					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-h; Hordeum vulgare / Golden Promise (Ref v1)					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	CABVVH010000001.1 Hordeum vulgare subsp, contig: chr1H, whole genome shotgun sequence	15701629	15701731	100bp_1H_F::100bp_1H_R	102	0::0
CHR-2	CABVVH010000002.1 Hordeum vulgare subsp, contig: chr2H, whole genome shotgun sequence	603634508	603634654	150bp_2H_F::150bp_2H_R	146	0::0
CHR-3	CABVVH010000003.1 Hordeum vulgare subsp, contig: chr3H, whole genome shotgun sequence	233140947	233141146	200bp_3H_F::200bp_3H_R	199	0::0
CHR-4	CABVVH010000004.1 Hordeum vulgare subsp, contig: chr4H, whole genome shotgun sequence	104163821	104164070	250bp_4H_F::250bp_4H_R	249	0::0
CHR-5	CABVVH010000005.1 Hordeum vulgare subsp, contig: chr5H, whole genome shotgun sequence	381777843	381778146	300bp_5H_F::300bp_5H_R	303	0::0
CHR-6	CABVVH010000006.1 Hordeum vulgare subsp, contig: chr6H, whole genome shotgun sequence	43844785	43845137	350bp_6H_F::350bp_6H_R	352	0::0
CHR-7	CABVVH010000007.1 Hordeum vulgare subsp, contig: chr7H, whole genome shotgun sequence	168460488	168460886	400bp_7H_F::400bp_7H_R	398	0::0
	Plex-a; Hordeum vulgare / Morex (Ref v3)					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-b; Hordeum vulgare / Morex (Ref v3)					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-d; Hordeum vulgare / Morex (Ref v3)					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-h; Hordeum vulgare / Morex (Ref v3)					
	CHR/Contig	5p coord.	3p coord.	Primer pair ID	fragment length	mismatch (F::R)
CHR-1	1H dna:primary_assembly primary_assembly:_pseudomolecules_assembly:1H:1:516505932:1 REF	30181355	30181457	100bp_1H_R::100bp_1H_F	102	0::0
CHR-2	2H dna:primary_assembly primary_assembly:_pseudomolecules_assembly:2H:1:665585731:1 REF	652788188	652788334	150bp_2H_F::150bp_2H_R	146	0::0
CHR-3	3H dna:primary_assembly primary_assembly:_pseudomolecules_assembly:3H:1:621516506:1 REF	246129369	246129568	200bp_3H_F::200bp_3H_R	199	0::0
CHR-4	4H dna:primary_assembly primary_assembly:_pseudomolecules_assembly:4H:1:610333535:1 REF	113766350	113766599	250bp_4H_F::250bp_4H_R	249	0::0
CHR-5	5H dna:primary_assembly primary_assembly:_pseudomolecules_assembly:5H:1:588218686:1 REF	408498106	408498409	300bp_5H_F::300bp_5H_R	303	0::0
CHR-6	6H dna:primary_assembly primary_assembly:_pseudomolecules_assembly:6H:1:561794515:1 REF	56727617	56727968	350bp_6H_F::350bp_6H_R	351	0::0
CHR-7	7H dna:primary_assembly primary_assembly:_pseudomolecules_assembly:7H:1:632540561:1 REF	186518611	186519009	400bp_7H_F::400bp_7H_R	398	0::0

Table S5- *In silico* prediction of chromosome-specific MPCR products in the genomes of *Triticum*, *Aegilops*, and *Hordeum* species

	Lack of PCR product or potential unspecific product					
	Plex-a; Triticum spelta					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1	1A :PGSBv2.0:1A:1:599764323:1 REF	5.42E+08	5.42E+08	100bp_F_1	99	0::0
CHR-2	2A :PGSBv2.0:2A:1:782685093:1 REF	7.25E+08	7.25E+08	150bp_F_2	149	0::0
CHR-3	3A :PGSBv2.0:3A:1:744407562:1 REF	3.82E+08	3.82E+08	200bp_F_3	199	0::0
CHR-4	4A :PGSBv2.0:4A:1:741299132:1 REF	1.92E+08	1.92E+08	250bp_F_4	248	0::0
CHR-5	5A :PGSBv2.0:5A:1:711661679:1 REF	35532102	35532401	300bp_F_5	299	0::0
CHR-6	6A :PGSBv2.0:6A:1:583494258:1 REF	5.69E+08	5.69E+08	350bp_F_6	349	1::0
CHR-7	7A :PGSBv2.0:7A:1:737453356:1 REF	4.37E+08	4.37E+08	400bp_F_7	398	0::0
	Plex-b; Triticum spelta					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1	1B dna:PGSBv2.0:1B:1:691313424:1 REF	1.14E+08	1.14E+08	100bp_F_1	99	1::0
CHR-2	2B dna:PGSBv2.0:2B:1:786410271:1 REF	6.46E+08	6.46E+08	150bp_F_2	149	1::0
CHR-3	3B dna:PGSBv2.0:3B:1:835583350:1 REF	1.58E+08	1.58E+08	200bp_F_3	206	0::0
CHR-4	4B dna:PGSBv2.0:4B:1:669032550:1 REF	4.33E+08	4.33E+08	250bp_F_4	249	0::0
CHR-5	5B dna:PGSBv2.0:5B:1:708205786:1 REF	66111660	66111959	300bp_F_5	299	0::0
CHR-6	6B dna:PGSBv2.0:6B:1:707105489:1 REF	1.5E+08	1.5E+08	350bp_F_6	349	0::0
CHR-7	7B dna:PGSBv2.0:7B:1:736349413:1 REF	6.12E+08	6.12E+08	400bp_F_7	398	0::0
	Plex-d; Triticum spelta					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1	1D dna:PGSBv2.0:1D:1:493357854:1 REF	1.12E+08	1.12E+08	100bp_F_1	99	0::0
CHR-2	2D dna:PGSBv2.0:2D:1:648139033:1 REF	28387099	28387248	150bp_F_2	149	0::0
CHR-3	3D dna:PGSBv2.0:3D:1:623221719:1 REF	1.13E+08	1.13E+08	200bp_F_3	199	0::0
CHR-4	4D dna:PGSBv2.0:4D:1:517040482:1 REF	1008868	1009117	250bp_F_4	249	0::0
CHR-5	5D dna:PGSBv2.0:5D:1:573398137:1 REF	1.27E+08	1.27E+08	300bp_F_5	299	0::0
CHR-6	6D dna:PGSBv2.0:6D:1:471251328:1 REF	19961379	19961728	350bp_F_6	349	0::0
CHR-7	7D dna:PGSBv2.0:7D:1:639162162:1 REF	1.4E+08	1.4E+08	400bp_F_7	401	0::0
	Plex-h; Triticum spelta					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Triticum turgidum subsp. durum					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1	1A dna:Svevo.v1:1A:1:585266722:1 REF	5.29E+08	5.29E+08	100bp_F_1	99	0::0
CHR-2	2A dna:Svevo.v1:2A:1:775448786:1 REF	7.19E+08	7.19E+08	150bp_F_2	176	0::0
CHR-3	3A dna:Svevo.v1:3A:1:746673839:1 REF	3.85E+08	3.85E+08	200bp_F_3	199	0::0
CHR-4	4A dna:Svevo.v1:4A:1:736872137:1 REF	1.9E+08	1.9E+08	250bp_F_4	249	0::0
CHR-5	5A dna:Svevo.v1:5A:1:669155517:1 REF	34459313	34459630	300bp_F_5	317	0::0
CHR-6	6A dna:Svevo.v1:6A:1:615672275:1 REF	6E+08	6E+08	350bp_F_6	349	0::0
CHR-7	7A dna:Svevo.v1:7A:1:728031845:1 REF	4.28E+08	4.28E+08	400bp_F_7	399	0::0
	Plex-b; Triticum turgidum subsp. durum					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1	1B dna:Svevo.v1:1B:1:681112512:1 REF	1.01E+08	1.01E+08	100bp_R_1	99	0::0
CHR-2	2B dna:Svevo.v1:2B:1:790338525:1 REF	6.38E+08	6.38E+08	150bp_F_2	149	1::0
CHR-3	3B dna:Svevo.v1:3B:1:836514780:1 REF	1.59E+08	1.59E+08	200bp_F_3	206	0::0
CHR-4	4B dna:Svevo.v1:4B:1:676292951:1 REF	4.25E+08	4.25E+08	250bp_R_4	249	0::0
CHR-5	5B dna:Svevo.v1:5B:1:701372996:1 REF	63374912	63375211	300bp_F_5	299	0::0
CHR-6	6B dna:Svevo.v1:6B:1:698614761:1 REF	1.46E+08	1.46E+08	350bp_F_6	349	0::0
CHR-7	7B dna:Svevo.v1:7B:1:722970987:1 REF	6.06E+08	6.06E+08	400bp_F_7	398	1::1

	Plex-d; Triticum turgidum					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-h; Triticum turgidum					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Triticum dicoccoides					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1	1A dna:WEWSeq_v.1.0:1A:1:593586810:1 REF	5.37E+08	5.37E+08	100bp_F_1	99	0::0
CHR-2	2A dna:WEWSeq_v.1.0:2A:1:775183943:1 REF	7.15E+08	7.15E+08	150bp_F_2	176	0::0
CHR-3	3A dna:WEWSeq_v.1.0:3A:1:754274518:1 REF	3.88E+08	3.88E+08	200bp_F_3	199	0::0
CHR-4	4A dna:WEWSeq_v.1.0:4A:1:726427787:1 REF	1.93E+08	1.93E+08	250bp_F_4	248	0::0
CHR-5	5A dna:WEWSeq_v.1.0:5A:1:700855599:1 REF	34051493	34051804	300bp_F_5	311	0::0
CHR-6	6A dna:WEWSeq_v.1.0:6A:1:621432051:1 REF	6.06E+08	6.06E+08	350bp_F_6	349	1::0
CHR-7	7A dna:WEWSeq_v.1.0:7A:1:727576108:1 REF	4.3E+08	4.3E+08	400bp_F_7	399	0::0
	Plex-b; Triticum dicoccoides					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1	1B dna:WEWSeq_v.1.0:1B:1:690537804:1 REF	1.04E+08	1.04E+08	100bp_F_1	99	0::0
CHR-2	2B dna:WEWSeq_v.1.0:2B:1:803365466:1 REF	6.46E+08	6.46E+08	150bp_F_2	149	1::0
CHR-3	3B dna:WEWSeq_v.1.0:3B:1:841096276:1 REF	1.6E+08	1.6E+08	200bp_F_3	206	0::0
CHR-4	4B dna:WEWSeq_v.1.0:4B:1:673896466:1 REF	4.44E+08	4.44E+08	250bp_F_4	249	0::0
CHR-5	5B dna:WEWSeq_v.1.0:5B:1:712180895:1 REF	68421782	68422081	300bp_F_5	299	0::0
CHR-6	6B dna:WEWSeq_v.1.0:6B:1:703217322:1 REF	1.55E+08	1.55E+08	350bp_F_6	349	0::0
CHR-7	7B dna:WEWSeq_v.1.0:7B:1:755408349:1 REF	6.35E+08	6.35E+08	400bp_R_7	398	0::0
	Plex-d; Triticum dicoccoides					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
Off-target	4B dna:chromosome chromosome:WEWSeq_v.1.0:4B:1:67	4177414	4177656	250bp_F_4	242	2::1
	Plex-h; Triticum dicoccoides					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
Off-target	1B dna:chromosome chromosome:WEWSeq_v.1.0:1B:1:69(	62056452	62056569	100bp_1H_	117	0::2

	Plex-a; Triticum urartu					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3	scaffold6217 dna:ASM34745v1:scaffold6217:1:106859:1 REF	26513	26712	200bp_F_3	199	1::0
CHR-4	scaffold44008 dna:ASM34745v1:scaffold44008:1:91215:1 REF	74071	74320	250bp_F_4	249	1::1
CHR-5						
CHR-6	scaffold22732 dna:ASM34745v1:scaffold22732:1:59272:1 REF	8291	8639	350bp_F_6	348	1::0
CHR-7	scaffold19683 dna:ASM34745v1:scaffold19683:1:78879:1 REF	12268	12667	400bp_F_7	399	0::0
	Plex-b; Triticum urartu					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-d; Triticum urartu					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-h; Triticum urartu					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Aegilops tauschii					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-b; Aegilops tauschii					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						

	Plex-d; Aegilops tauschii					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1	1D dna:Aet_v4.0:1D:1:502330251:1 REF	1.15E+08	1.15E+08	100bp_F_1	99	0::0
CHR-2	2D dna:Aet_v4.0:2D:1:651661114:1 REF	28450372	28450521	150bp_F_2	149	0::1
CHR-3	3D dna:Aet_v4.0:3D:1:627182665:1 REF	1.06E+08	1.06E+08	200bp_F_3	199	0::0
CHR-4	4D dna:Aet_v4.0:4D:1:526018785:1 REF	1021383	1021632	250bp_F_4	249	0::0
CHR-5	5D dna:Aet_v4.0:5D:1:577375663:1 REF	1.3E+08	1.3E+08	300bp_R_5	300	0::0
CHR-6	6D dna:Aet_v4.0:6D:1:496019527:1 REF	19960208	19960557	350bp_F_6	349	1::0
CHR-7	7D dna:Aet_v4.0:7D:1:644716137:1 REF	1.4E+08	1.4E+08	400bp_F_7	401	0::0
	Plex-h; Aegilops tauschii					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-a; Hordeum vulgare subsp. spontaneum / Wild barley					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-b; Hordeum vulgare subsp. spontaneum / Wild barley					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-d; Hordeum vulgare subsp. spontaneum / Wild barley					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-h; Hordeum vulgare subsp. spontaneum / Wild barley					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1	OU015701.1 Hordeum vulgare subsp. Spontaneum	20098912	20099014	100bp_1H_	102	0::0
CHR-2	OU015702.1 Hordeum vulgare subsp. spontaneum	6.75E+08	6.75E+08	150bp_2H_	146	0::0
CHR-3	OU015703.1 Hordeum vulgare subsp. Spontaneum	2.53E+08	2.53E+08	200bp_3H_	199	0::1
CHR-4	OU015704.1 Hordeum vulgare subsp. spontaneum	1.19E+08	1.19E+08	250bp_4H_	246	0::1
CHR-5	OU015705.1 Hordeum vulgare subsp. spontaneum	4.19E+08	4.19E+08	300bp_5H_	303	0::0
CHR-6	OU015706.1 Hordeum vulgare subsp. spontaneum	54380128	54380479	350bp_6H_	351	0::0
CHR-7	OU015707.1 Hordeum vulgare subsp. spontaneum	1.84E+08	1.84E+08	400bp_7H_	398	0::0

	Plex-a; Hordeum marinum					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-b; Hordeum marinum					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-d; Hordeum marinum					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2						
CHR-3						
CHR-4						
CHR-5						
CHR-6						
CHR-7						
	Plex-h; Hordeum marinum					
	CHR/Contig	5p coord.	3p coord.	Primer pair	fragment length	mismatch (F::R)
CHR-1						
CHR-2	CM039673.1, whole genome shotgun sequence	5.65E+08	5.65E+08	150bp_2H_	145	0::1
CHR-3						
CHR-4						
CHR-5	CM039676.1, whole genome shotgun sequence	2.71E+08	2.71E+08	300bp_5H_	315	2::1
CHR-6						
CHR-7						

Table S6. Sequence of primers and guide RNA used in this study

Primer	Sequence (5'-3')	Utilization
Ubi_Det_F	AACCAGATCTCCCCCAAATC	Zea Mays Ubi1 promoter detection primer
Ubi_Det_R	AAACCAAACCCTATGCAACG	
gRNA2_insert_F	GGCGCGGCACAAGAACGCGCTGG	To insert guide RNA2 into the plasmid
gRNA2_insert_R	AAACCCAGCGCGTTCTTGTGCCG	
TaMLOABD_Seq_F	CTCCGTCCTCCTGGAGCACGCG	For PCR/RE assay
TaMLOABD_Seq_R	GTGACGGCGAGCAGCAGCGAG	
Cas9_Det_F	GCCTACCACGAGAAGTACCCTAC	Cas9 detection primer
Cas9_Det_R	GGTCATCGTCGTATGTGTCCTTG	
Hyg_Det_F	CGGAAGTGCTTGACATTGGGGAG	Hygromycin detection primer
Hyg_Det_R	GCATCAGCTCATCGAGAGCCTG	
Tamlosg2	GCGGCACAAGAACGCGCTGG CGG	sgRNA used in this study
sgMLO-A1	CCG TCACGCAGGACCCAATCTCC	Wang et al., 2014

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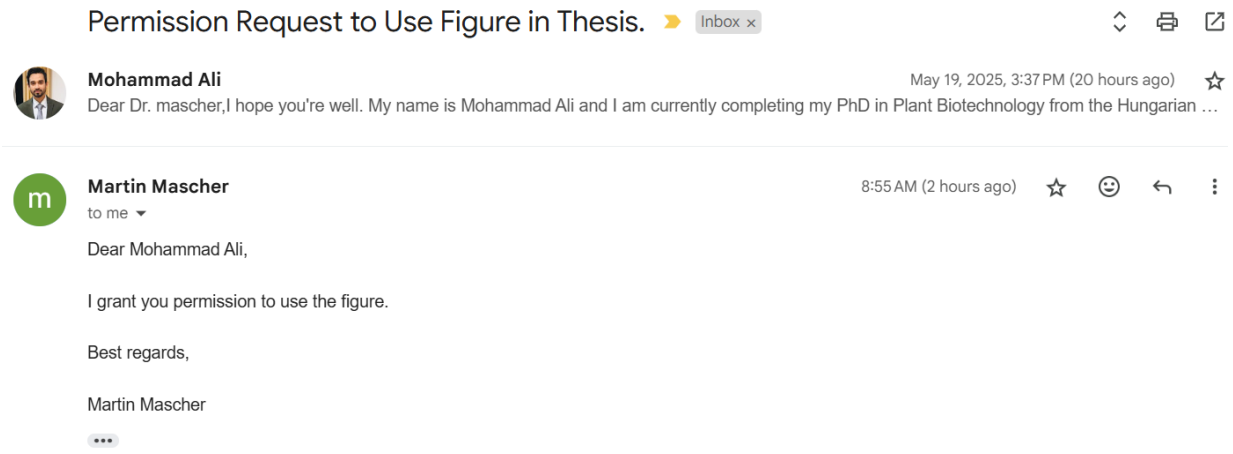

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